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Doctoral Thesis

Integration of stability and activity for *Candida antarctica* lipase B and its covalent immobilization on a modified sol-gel matrix for biodiesel production

February 2015

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Integration of stability and activity for *Candida antarctica* lipase B and its covalent immobilization on a modified sol-gel matrix for biodiesel production

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Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Seoul National University

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School of Chemical and Biological Engineering

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ABSTRACT

Lipases play a vital role in various processes. Lipase-mediated processes can be done in mild reaction conditions requiring less energy, faster reaction rates, a cheaper starting materials can be utilized since lipases are selective and wastewater treatment/downstream processing pose no problem for lipases are biodegradable. Even though the use of lipase provides many advantages, their industrial application is hindered due to their expensive cost. In addition, lipases have low activity with long chain fatty acids as substrate and they are easily inhibited by short chain alcohol which leads to their shorter life span when applied as biocatalyst. A lipase with improved characteristics is in demand for a biochemical process to be economically feasible.

In this study, structural flexibility modulation and immobilization were carried out in order to enhance the functionality of *Candida antarctica* lipase B (CALB). The dynamics, structure configuration and functional groups of CALB were used to obtain an enzyme that shows higher activity and robustness. The dynamics of CALB in terms of its flexibility was modulated to improve the activity and stability. Structure configuration was also considered to maximize the effect and impact of mutation. Functional groups specifically exposed lysine residues were exploited to immobilize CALB on a modified sol-gel matrix.

Both stability and activity improvements were incorporated in *Candida antarctica* lipase B (CALB) through multiple-site mutagenesis. CALB was divided into two different regions to optimize its performance. Modulating the flexibility within the substrate-binding region and the hydrophilic solvent-affecting region enhanced the catalytic activity and organic solvent stability of CALB, respectively. Combining the mutation sites from the substrate-binding region and from the hydrophilic solvent-affecting region yielded an enzyme (V139E,A92E) with improved functionality.

The use of modified sol-gel matrix to immobilize CALB was investigated. Free hydroxyl groups on the matrix surface were exploited to covalently immobilize the enzyme. Based from the results, incorporating hydrophobic sol-gel precursor (ethyltrimethoxysilane) enhanced enzyme activity. An enzyme activity of 192.02 U/g beads with 80.88% attachment was obtained. At alkaline pH, immobilization yield of enzyme increased. The attachment of enzyme on the surface of the matrix was confirmed by scanning electron microscope images. Covalently immobilized CALB on sol-gel supports yielded higher thermal stability with 2.7 times higher half-life compared with soluble enzymes at 60^{o}C. This enzyme immobilization system retained the enzyme residual activity even for repetitive use.

The resultant enzyme with enhanced functionality was covalently immobilized on the modified sol-gel matrix and its performance on biodiesel production was tested. The biodiesel production was approximately 3.7 times higher using the immobilized double mutant (V139E,A92E) enzyme compared with the immobilized wild type CALB. Optimization on the immobilized enzyme system using the double mutant (V139E,A92E) can be further investigated to further increase the biodiesel production. The technique applied in this study can also be extended to other lipases.

Keywords: *Candida antarctica* lipase B, enzyme flexibility, enzyme stability, mutagenesis, sol-gel matrix, covalent immobilization

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Chapter 1

Introduction

1.1 Backgrounds

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are ubiquitous enzymes of physiological and industrial significance. Lipases are applied in organic chemical processing, detergent formulations, synthesis of biosurfactants, dairy industry, paper manufacture, bioenergy production, and pharmaceutical processing. Due to their substantial significance in biochemical processes, lipases remain a subject of intensive investigation. Research on lipases is focused on structural characterization, elucidation of mechanism of action, kinetics, sequencing and cloning of lipase genes, and general characterization of performance [Alberghina et al., 1991; Bornscheuer, 2000]. Even though lipases have a wide-range of application, relatively little work has been done on the development of robust lipase bioreactor systems for commercial use.

In order for lipases to be utilized in large-scale processes, they should possess robustness, high catalytic efficiency as well as reusability. Different approaches are being considered for enzyme stabilization. The first method is to screen better enzymes among the pool of qualified enzymes or environmental DNA using metagenomic techniques. This type of method could be laborious since there is a large number of enzymes present in the environment. The second method is the modification of the enzyme's environment such as

lyophilization, additing additives, and an effective enzyme immobilization technique. This is the traditional approach for stabilizing the enzyme. The third method is enzyme engineering, wherein the enzyme is genetically modified through mutagenesis in order to come up with a robust enzyme.

The structural flexibility of the enzyme has been associated with its catalytic function. As presented from the study of psychrophilic *Candida antarctica* lipase B (CALB), it is perceived that the high flexibility of CALB made it adaptive and more active at low temperatures. Modulating the flexibility of the helix region surrounding the active site of CALB enhances the activity of the enzyme [Hong and Yoo, 2013]. On the other hand, it was reported that when an enzyme is mutated such that it loses its flexible motion, the enzyme underwent conformational fluctuation change and decrease in catalytic activity [Bhabha et al., 2011]. Enzyme rigidification can stabilize the enzyme. Xie et al., [2014] enhanced the kinetic stability of CALB by increasing rigidity within the active site. However oftentimes, the enzyme activity is often compromised by the rigidification of the enzyme structure [Arnold et al., 2001] such that a trade-off between activity and stability is normally considered when designing an enzyme.

Aside from catalytic efficiency and stability offered by enzymes, it is also important to easily separate the enzyme from the product and reuse it for the subsequent reactions. This will potentially lower the production cost, simplifies enzyme application, and supports a reliable and efficient reaction technology thus making the enzyme attractive as a catalyst for industrial use. Enzyme immobilization provides these advantages. The characteristics of the immobilized enzyme systems are governed by the properties of both enzyme and the support material. The specific interaction between the support and the enzyme provides an immobilized enzyme with distinct chemical, biochemical, mechanical and kinetic properties [Tischer and Wekekind, 1999]. Lipases can be immobilized through adsorption, entrapment, cross-linking and covalent attachment.

1.2 Research Objectives

Candida antarctica lipase B has many applications. However, its industrial application is hampered due to its expensive cost and activity depletion upon continuous use. In order to solve these problems, CALB must have higher catalytic activity, stability and reusability. The goal of this study is to enhance the functionality of CALB. Specifically this study aims to:

(a) Modulate the flexibility of CALB on the substrate binding region to enhance the activity,

(b) Rigidify the hydrophilic solvent affecting region to enhance the enzyme stability,

(c) Determine the mutation site combination to integrate both stability and activity enhancement on CALB,

(d) Develop an enzyme immobilization system, and

(e) Determine the feasibility of the enzyme immobilization system being developed in the biodiesel production.

Enzyme mutagenesis and immobilization was being proposed in this study to improve the catalytic performance of CALB. Enzyme dynamics, through modulation of flexibility or rigidity of the enzyme moiety by double mutation, was exploited in order to improve the properties of CALB. Modified sol-gel matrix was used to covalently immobilized CALB. Stability and reusability was considered to increase the lifespan of the enzyme.

Chapter 2

Literature Survey

2.1 Microbial Lipases

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are important biocatalysts because of their excellent physiological and biochemical properties. They are produced by several plants, animals, and microorganisms. Microbial lipases have gained special industrial attention due to their ability towards extremes of temperature, pH, and organic solvents, and chemo-, regio-, and enantioselectivity [Verma et al., 2012]. Lipase can present molar mass ranging from 20 to 75 kDa, enzymatic activity at temperatures from 27 to 70^{o}C and pH between 4-9, depending on the source. They are hydrolyses that act on carboxyl ester bonds in triglycerides to yield fatty acids and glycerol. Lipases catalyzed this at the lipid-water interface. They have a central L-sheet like structure with an active site consisting of serine on the nucleophilic elbow placed in a channel. This channel is covered by a peptide lid. When lipase comes in contact with a lipid-water interface this lid undergoes conformational changes exposing the active site for substrate accessibility [Villeneuve et al., 2000].

Intracellular and extracellular lipases are the two major categories of enzymatic biocatalyst. For intracellular lipase, the enzyme remains either inside the cell or in the cell-producing walls where the cells need to be disrupted to obtain the enzyme. On the hand, extracellular lipase are induced in the live-

producing microorganism broth and then purified. Microorganisms such as *Mucor miehei*, *Rhizopus oryzae*, *Candida antarctica*, *Candida rugosa*, *Aspergillus niger*, and *Pseudomonas cepacia* are examples of lipase producing microorganisms. Most commercial lipolytic preparations are composed by a mixture of various isozymes, in different proportions, such as those obtained from *Candida rugosa*, *Candida antarctica*, *Rhizopus niveus*, and *Chromobacterium viscosum* [Ribeiro et al., 2011]. Each isoform will have different properties such as specificity, stereoselectivity and substrate preference and molar mass.

Substrate specificity of lipase is of great importance in the selection and usage of lipase in industrial processes. Lipases can be divided into three groups based on their specificity as 1,3-specific lipases, nonspecific lipases and fatty acid-specific lipases.

(a) 1,3-specific lipases (*e.g.* from *Aspergillus niger*, *Rhizopus oryzae* and *Mucor miehei*): They release fatty acids from positions 1 and 3 of a glyceride and hydrolyze ester bonds in these positions. Thus, the use of this kind of lipase can give rise to biodiesel yield of above 90% under appropriate conditions [Antczak et al., 2009].

(b) Nonspecific lipases (*e.g.* from *Candida rugosa*, *Pseudomonas* sp and *Chromobacterium viscosum*): They do not distinguish between positions of ester bonds to be cleaved. The product obtained from the catalysis of these lipases is similar to those produced by chemical methods but with less thermodegradation, due to low reaction temperature.

(c) Fatty acid-specific lipases (*e.g.* most studied example *Geotrichum candidum*): This kind of lipase acts on the hydrolysis of esters that have long-chain fatty acids with double bonds in *cis* position on carbon 9 [Uhlig, 1998].

Although the application of lipases in the food, detergent, pharmaceutical, leather, paper and cosmetics are very promising, their industrial uses still remain limited due to their high production costs, low amounts for commercialization and low performance if used continuously for some lipase-mediated processes. Researches are geared towards increasing the lifespan, catalytic activity and broadening and exploring new applications for lipases. Several approaches have been investigated for this purpose such as genetic engineering, mutation of the lipase itself, enzyme immobilization, chemical modification, physical treatment and reaction and reactor engineering to enhance their catalytic capability.

2.2 Functional Motions of Enzymes

Enzymes are dynamics and flexible molecules that are in constant motion. Upon variations in the environment, the enzyme backbone can undergo significant conformational changes. This enzyme motion can range from molecular flexibility to large-scale conformational transition that has an essential role in many biochemical processes. Internal motions of the enzymes have different amplitudes and the frequencies range over different time scales, see Fig. 2.1. The motions which affect the enzyme structure include bond vibrations at the time scale of femto- to pico-seconds, side-chain rotations at the time scale of nanoseconds, and more complicated motions of larger time scales such as motion of flexible termini and loops, large concerted domain motions, and conformational adjustment upon substrate binding [Kokkinidis et al., 2012].

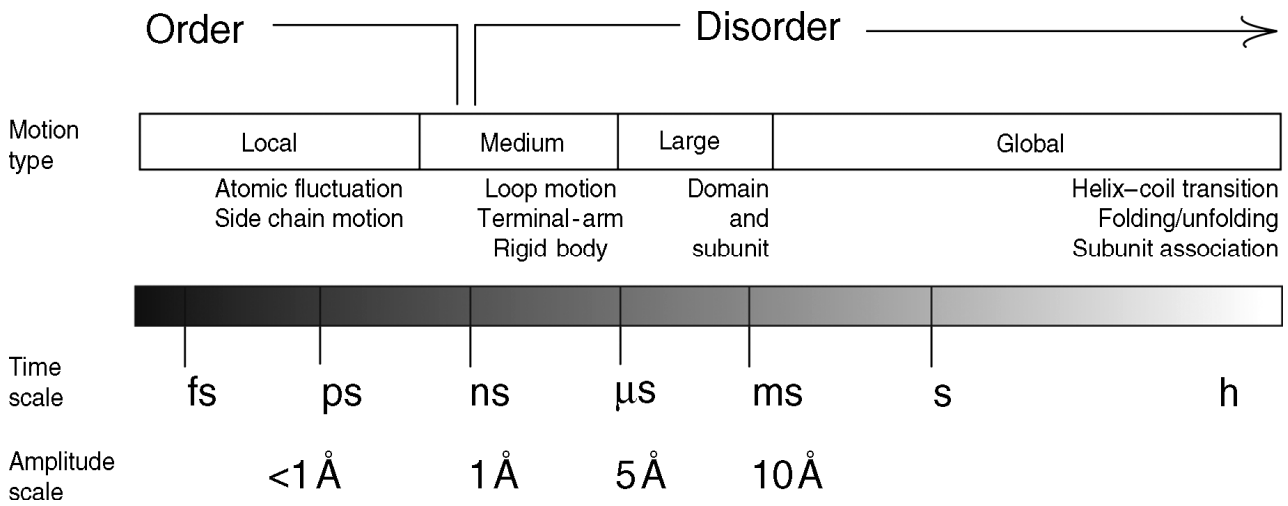

Fig. 2.1 Range of enzyme dynamics and structural observation [Gu and Hilser, 2009]

The structural flexibility that enables enzyme motion has been associated with various biological processes such as molecular recognition and binding, protein-protein interactions, and complicated processes such as signal transduction and assembly of multiple machines [Gazi et al., 2009; Teilum et al., 2011]. The ability of enzymes to adopt to these different conformations is vital for the efficiency of the catalytic processes. The flexibility of the active site is considered as a requirement for reduction of free energy barrier in which enzymatic reaction is accelerated. During the catalytic cycle, the enzyme molecule passes through different states and each state is associated with a different active site conformation. Rapid transition between the different conformational states is therefore mandatory for the maximum enzyme activity.

There are four categories of enzyme flexibility: (1) low-B-factor ordered regions, (2) high-B-factor ordered regions, (3) short disorder regions, and (4) long disordered regions. The B-factor (temperature factor) has been used to measure residue flexibility of folded proteins. It reflects the uncertainty in atom positions in the model and often represents the combined effects of thermal vibrations and static disorder [Rhodes, 1993]. Radivojac et al., [2004] studies the amino acid compositions of the four categories and found that they are significantly different from each other, with high-B-factor ordered and short disordered regions being the most similar pair. The high-B-factor (flexible)

ordered regions are characterized by a higher average flexibility index, higher average hydrophilicity, higher average absolute net charge, and higher total charge than disordered regions.Low-B-factor regions have enriched in hydrophobic residues and depleted in the total number of charged residues compared to the other three categories. An extreme manifestation of enzyme flexibility is the molten globule state and the intrinsically disordered proteins. A disordered segment does not have a well-defined folded structure that can be determined by experimental techniques such as X-ray crystallography or NMR [Kuznetsov, 2008]. Intrinsically disordered proteins can perform biological functions despite lacking of well-defined tertiary structures under physiological conditions.

Candida antarctica lipase B (CALB) is the most widely studied psychrophilic lipase with a great number of registered patents and various applications [Joseph et al., 2008]. CALB is applied industrially due to its high enantioselectivity, wide array of substrates, thermal stability, and stability in organic solvents. CALB is a globular protein with an α/β hydrolase fold consisting of 317 amino acids and a molecular weight of 33kDa. Its active site consists of the catalytic triad: S105, D187 and H224. In contrast to most lipases, CALB has no lid covering the entrance to the active site and shows no interfacial activation [Martinelle et al., 1995].

Trodler and Pleiss [2008] investigated the structure and flexibility of CALB in organic solvents. They reported that the overall flexibility of CALB decreases in organic solvents in the order methanol, isopentane, chloroform, toluene, and cyclohexane. The degree of decreased flexibility is different for each solvent but the regions with higher flexibility are the same in every solvent. There are 5 surface elements that undergo changes in flexibility and are solvent-dependent as shown in Fig. 2.2: a short α-helix (α5, residues 139-150), a long α-helix (α10, residues 266-289) which form the entrance to the active site and three surface loops (residues 26-30, 92-97 and 215-222). The reduced flexibility of CALB in non-polar solvents is not only a consequence of the interaction between organic solvent molecules and the protein, but also due to the interaction with the enzyme-bound water and its exchange on the surface.

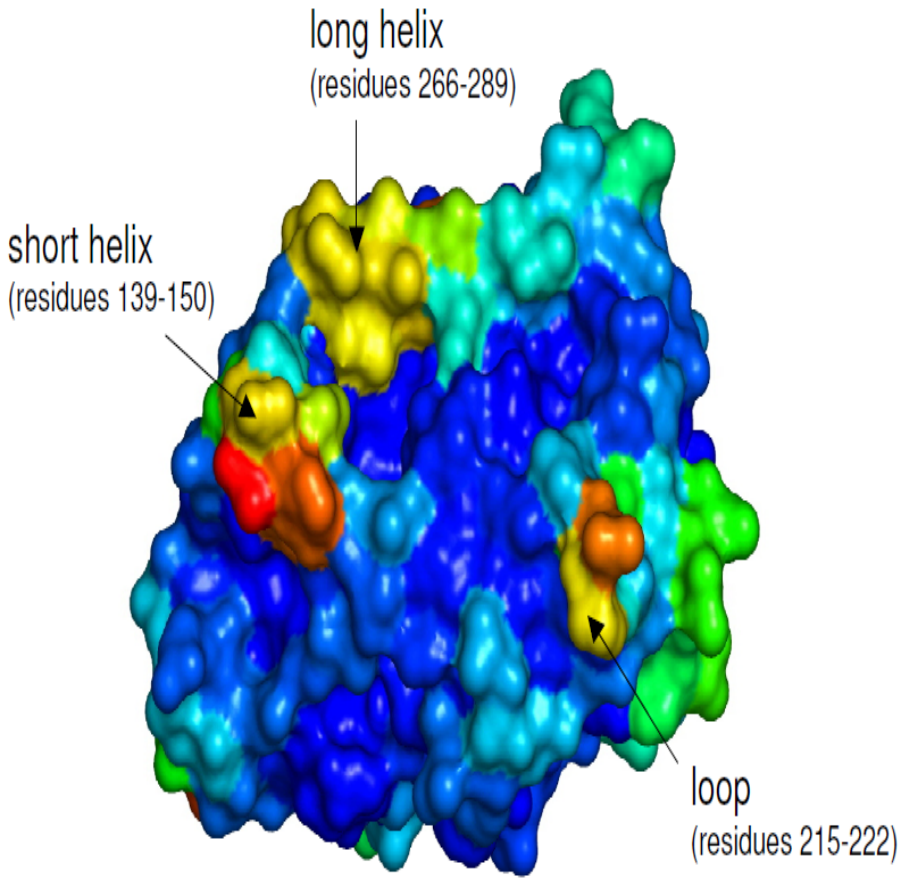

Fig. 2.2 Flexible regions in CALB. Regions with high flexibility are labeled; regions colored blue have low flexibility and regions colored red have high flexibility [Trodler and Pleiss, 2008]

Ganjalikhany et al., [2012] performed molecular dynamics (MD) simulations of psychrophilic CALB for tracing open-closed conformations under different temperatures. Root mean square fluctuations (RMSF) of C_α revealed significant local flexibility with distinct differences at 5, 30 and 50^oC, for residues 142-147 (located at α5 helix). The extent of flexibility at α5 is much higher at 5^oC compared to the other temperatures. The starting open conformation became closed immediately at30 and 50^oC during 60 ns simulation. Flexibility of α10 is increased with lower extent as temperature is elevated. This flexibility of CALB at lower temperature is localized to its active site area, while the global stability of enzyme is not affected by temperature significantly possibly due to the three disulfide bonds that help stabilized the enzyme at both low and high temperatures. It is suggested that the functional motions needed for lypolytic activity of CALB is constructed from short-range movement of α5, accompanied by long-range movement of the domains connected to the lid region.

Gruber and Pleiss [2012] presented through MD simulation in full atomistic detail that CALB attaches and binds to a hydrophobic tributyrin-water interface via three hydrophobic anchor regions defined by Leu 147, Leu 219, and Val 272 surrounding the active site. These three regions trigger the reorientation of the protein via hydrophobic interactions even when the enzyme

impacts at the surface in a non-optimal orientation. The flexible helix α5 undergoes a movement of 7.5Å towards the substrate layer during the binding process.

2.3 Enzyme Immobilization

Immobilization technology for enzyme provides an effective method to circumvent the problems associated with the poor stability, reusability and high cost of single enzyme use that limits the enzyme application for industrial processes. Immobilized enzyme is defined as the physical confinement or localization of enzyme in a certain defined region of space with retention of its catalytic activity and in which the immobilized system can be used repeatedly [Jegannathan et al., 2008]. The comparison of free enzymes and immobilized enzymes is given in Table 2.1. The methods for enzyme immobilization can be classified as adsorption, entrapment, cross-linking and covalent bonding. The choice of method and support material is an important factor to be considered to obtain an efficient enzyme.

To evaluate the performance of the immobilization system, the following parameters are usually considered:

(a) Catalytic activity: This is important to have a high specific activity in order for the enzyme to be applied efficiently. This parameter is influenced by the method of immobilization.

(b) Final yield: The amount of enzyme immobilized on the support is important to maximize the enzyme load. Since enzyme is expensive the enzyme loading should be considered for industrial purposes.

(c) Operational stability: This is often of crucial importance for industrial scale applications. Stability is required to achieve high productivity [Adlercreutz, 2013].

Table 2.1 Comparison of free and immobilized enzyme [Zhang et al., 2012]

Characteristics	Free Enzyme	Immobilized Enzyme
Price	High	Low
Efficiency	Low	High
Activity	Unstable	Stable
Reusability and recovery	Not possible	Possible
Tolerance to temperature, pH etc.	Low	High
Separation from the substrate	Difficult	Easy
Recovery from the product	Difficult	Easy

2.3.1 Adsorption

Adsorption is the adhesion of the enzyme on the surface of the carrier by weak forces, such as van der Walls, ionic interactions, hydrophobic interactions or dispersion forces [Jegannathan et al., 2008]. The method is simply mixing an aqueous solution of the enzyme with the desired support/carrier material for a period of time and washing away the excess enzyme from the immobilization system [Costa et al., 2005]. Adsorption is a reversible enzyme immobilization method. It is the most widely used method for immobilization due to its simplicity and commercial advantages. It offers easy preparation at mild condition, low cost, no need for additional chemicals, support can be recovered for repeated use and high activity [Fukuda et al., 2001].

Novozyme® 435, a commercial lipase, is obtained by immobilization of *Candida antarctica* lipase B (CALB) on acrylic resin and considered as a good catalyst since it provides biodiesel yield higher than 90% with vegetable oil or waste oil as feedstock [Tan et al., 2010]. Hernandez et al. [2011] evaluated two commercial porous styrene-divinylbenzene beads (Diaion HP20LX and MCI GEL CHP20P) as supports to immobilize lipase B from *Candida antarctica* (CALB). They reported that MCI GEL CHP20P permits a very high loading capacity around 110mg of CALB/wet g of support compared to the 50mg

obtained using decaoctyl Sepabeads. Other types of carriers used in adsorption are celite, polypropylene and textile membrane [Shah et al., 2004; Salis et al., 2008; Li et al., 2010]. Although adsorption as method for immobilization offers many advantages, the main disadvantage is the desorption of the lipase from the support due to the weak interactions between the enzyme and the support.

2.3.2 Covalent Immobilization

In covalent immobilization, covalent bonds between the functional groups of support surface and active nonessential amino acid residues on the enzyme surface will be formed [Costa et al., 2005]. This strong interaction between the support and the enzyme makes the enzyme more thermally and operationally stable. It is necessary that the immobilization reaction to form the chemical bond between the enzyme and the support should be carried out under mild conditions because vigorous reaction condition can destroy enzyme active conformation. In the case of lipases, it might be necessary to immobilize the enzyme under conditions favoring the closed form to protect the active site. Several support materials have been utilized for covalent immobilization such as the commercially available Eupergit®, hydrophobic polypeptides, nylon fibers and natural polymers.

Mendes et al., [2010] investigated the multipoint covalent immobilization of lipase on chitosan hydride hydrogels. The obtainedsupports were chemically modified with 2,4,6-trinitrobenzene sulfonic acid (TNBS) to increase support hydrophobicity, followed by activation with different agents such as glycidol (GLY), epichlorohydrin (EPI), and glutaraldehyde (GLU). The chitosan-alginate hydrogel, chemically modified with 2,4,6-trinitrobenzene sulfonic acid(TNBS), provided derivatives with higher apparent hydrolytic activity and thermal stability, being up to 45-fold more stable than soluble lipase. Using two immobilized lipases with complementary position specificity instead of just a single enzyme can enhance biodiesel production. Lipase from *Rhizopus oryzae* and *Candida rugosa* was covalently bound to the silica support. Under optimum conditions, the conversion rate of degummed crude canola oil to fatty acid methyl esters was higher compared to the once obtained from the free enzyme mixture [Jang et al., 2012].

2.3.3 Cross-linking

Cross-linking is defined as the interaction of a three dimensional network within enzyme, coupling agent, and the support material. Although strong interaction can be made between the enzyme and the support material, cross-linking conditions are intense and the immobilized lipase might show

lower activity. Enzymes can also be immobilized via cross-linking enzyme molecules with a bifunctional cross-linking agent obtaining 100% active enzyme, carrier free. Glutaraldehyde is normally the cross-linking agent of choice as it is inexpensive and readily available. However, glutaraldehyde is highly reactive and of small size that can easily penetrate the interior of the enzymes eventually inactivating them. Bulky polyaldehydes are now also being used as cross-linker.

Cross-linked enzyme crystals (CLECs) is an industrial biocatalyst commercialized by Altus Biologics. It shows operational stability, controllable particle size and ease of recycling. However, crystallization of enzyme is laborious and high purity of the enzyme is essential. Thus, cross-linked enzyme aggregates (CLEAs) is introduced where in it combines the purification and immobilization into a single unit operation. Pan et al., [2011] cross-linked enzyme co-aggregates of *Serratia marcescens* lipase using polyethyleneimine as co-precipitant and glutaraldehyde as cross-linking reagent. The CLEAs showed excellent operational stability in repeated used in aqueous-toluene biphasic system for asymmetric hydrolysis of trans-3-(4'-methocyphenyl)glycidic acid methyl ester (MPGM), without significant inactivation after 10 rounds of repeated use.

2.3.4 Entrapment

Entrapment method is based on capturing the enzyme within a polymer network that retains the enzyme but allows the substrate and product to pass through [O'Driscoll, 1976]. The enzyme in this technique is not attached to the polymer, its free diffusions is simply restrained. Entrapment can be divided in two categories: gel or fiber entrapment and microencapsulation. The method for entrapment is fast, cheap and usually involves mild conditions. However, the biggest drawback of entrapment is the mass transfer limitation since the support can act as a barrier for the substrate and product transport so that the enzyme is only effective for low molecular weight substrates [Murty et al., 2002].

A number of support materials have been investigated for the entrapment method such as alginate, celite, activated carbon, organic polymers and sol-gel matrix. A traditional way to immobilize an enzyme through encapsulation is to carry out polymerization and cross-linking in a solution containing monomers and the enzyme, so that the enzyme will be entrapped in the polymer network. Entrapment of enzyme in organic polymer such as poly-*N*-isopropylacrylamide involves mixing the aqueous enzyme solution with a pre-formed polymer particles followed by an increase in temperature (~50^{o}C) which caused a polymer shrinkage and entrapped the enzyme. CALB has been

immobilized in micron-sized poly-*N*-isopropylacrylamide hydrogel particles, and the particles were transferred to propanol and subsequently to hexane [Gawlitza et al., 2012]. In this method the enzyme would leak out of the particles if placed in water, while the enzyme remained inside the particles in the organic medium as it is not soluble. Sakai et al., [2008] studied the entrapment of *Rhizopus arrhizus* lipase in electrospunpoly(vinyl alcohol) fibers having a diameter of about 1μm. The initial rate of transesterification of the entrapped lipase is 5.2-times higher than the non-treated one which made it applicable for transesterification reaction in a flow-through reactor.

2.4 Sol-Gel Material for Enzyme Immobilization

The investigations of Ebelmen [1846] and Graham [1864] on silica gels lead to the hydrolysis of tetraethyl orthosilicate (TEOS), $Si(OC_2H_5)_4$, under acidic conditions that produced SiO_2 in the form of a glass-like material. Monolithic optical lenses and fibers could be drawn from the viscous liquid. However, long periods of drying were needed to avoid the silica gels fracturing into fine powders. Considerable interest came when very high levels of chemical homogeneity in colloidal gels and synthesizes of large number of novel ceramic powder oxide compositions (*e.g.* Al, Si, Ti and Zr), that could not be made during traditional ceramic methods, are needed. The work of Iler [1955]

led to the commercial development of colloidal silica powders and Stober et al., [1968] extended this finding to control both the morphology and size of the powders using ammonia as a catalyst for TEOS producing the so-called Stober spherical silica powder.

2.4.1 Sol-Gel Process Steps

Sols are defined as dispersions of colloidal particles in a liquid. The interconnected, rigid network with pores of submicrometer dimensions and polymetic chains whose average length is greater than a micrometer is referred to as *gels*. There are three approaches to make sol-gel monoliths: (Method 1) gelation of a solution of colloidal powders; (Method 2) hydrolysis and polycondensation of alkoxide or nitrate precursors followed by hypercritical drying of gels; and (Method 3) hydrolysis and polycondensation of alkoxide precursors followed by aging and drying under ambient conditions [Hench and West, 1990].

A silica gel may be formed by network growth from an array of discrete colloidal particles as in method 1 or by formation of an interconnected 3-D network by the simultaneous hydrolysis and polycondensation of an organometallic precursor as of methods 2 and 3. When the liquid in the pores of

the material is removed at or near ambient pressure by thermal evaporation (drying used in methods 1 and 3) and shrinkage occurs, the monolith is termed a *xerogel*. On the other hand, when the pore liquid is removed as a gas phase from the interconnected solid gel network under hypercritical conditions (critical-point drying used in method 2), there will be no shrinkage and low density *aerogel* is formed.

The formation of sol-gel silica monoliths involves 7 steps as follows:

Step 1: *Mixing*. In method 1, mechanical mixing of colloidal particles in water at a certain pH that prevents precipitation is done to form a suspension of colloidal powders, sols. For methods 2 and 3, the liquid alkoxide precursor, $Si(OR)_4$, is hydrolyzed by mixing with water. The chemical reaction in the sol-gel formation is presented in Fig. 2.3.

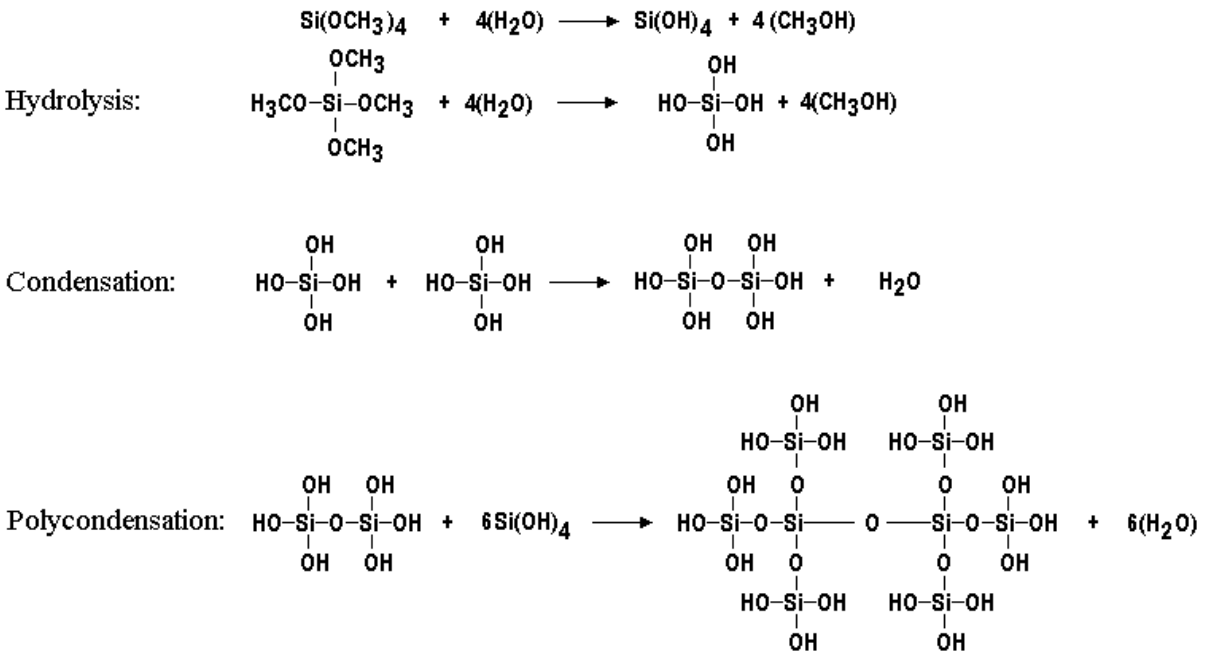

Fig. 2.3 Chemical reactions involved in sol-gel formation

The liquid precursor, $Si(OR)_4$, where R is CH_3 or C_2H_5 is hydrolyzed with water forming hydrated silica tetrahedral and alcohol as by-product. The hydrated silica tetrahedral will then interact in a condensation reaction forming ≡S–O–S≡ bonds. Linkage of additional ≡S–OH tetrahedral occurs as polycondensation reaction and eventually results in a SiO_2 network. The by-products alcohol and water from the reaction remains in the pores of the network.

Step 2: *Casting*. The sol can be cast into a mold since it is a low-viscosity liquid. The gel must not adhere into the mold, thus the mold of choice must be considered.

Step 3: *Gelation*. Three-dimensional network will be formed when the colloidal particles and condensed silica species linked together. During this stage, the viscosity increases and a solid object results in the shape of the mold.

Step 4: *Aging*. Aging involves maintaining the cast object for a period of time completely immersed in the liquid. During this stage, polycondensation continues which increases the thickness of interparticle necks and decreases the porosity of the material. Aging also strengthen the gel to resist cracking during drying.

Step 5: *Drying*. The liquid in the interstices of the network will be removed during this step. The gel is considered dried when the physically absorbed liquid is completely removed. This occurs between 100-180^{o}C.

Step 6: *Chemical Stabilization*. The removal of surface silanol (S–OH) bonds from the pore network results in a chemically stable ultraporous solid. Many chlorine compounds can completely react with surface hydroxyl groups to form hydrochloric acid, which then desorbs from the gel at a temperature ranging from 400-800^{o}C.

Step 7: *Densification*. This step occurs when the porous gel is heated at high temperature. The pores will be eliminated and the density ultimately becomes equivalent to fused silica. Densification of a gel network occurs between 1000 and 1700^{o}C [Hench and West, 1990].

2.4.2 Sol-Gel Entrapment

Silica-based carriers are widely used for enzyme immobilization. The entrapment of enzymes is based on the sol-gel technique to generate silica matrices by acid or base-catalyzed hydrolysis of hydrolysable silane compounds such as TEOS. Reetz et al., [1995] efficiently immobilized lipases by entrapment in hydrophobic sol-gel matrix. They presented that enhanced enzyme activity correlates with enhanced hydrophobicity in the silicon oxide matrix. Higher thermal stability and activity appear to results from multipoint interactions through hydrogen bonding as well as ionic and hydrophobic interactions (van der Waals) as shown in Fig. 2.4. Hydrophobic interactions can

result in a type of interfacial activation. The lipase may be conformationally arrested in the matrix in a "lid-opened" and thus in its active form [Reetz, 1997].

Chen and Lin [2003] entrapped *Candida rugosa* in a hybrid organic-inorganic powder prepared by acid-catalyzed polymerization of tetramethoxysilane (TMOS) and alkyltrimethoxysilanes. The percentage of protein immobilized was 95% and the resulting lipase specific activity was 59 times higher than that of a non-immobilized lyophilized lipase. The mechanical, operational and thermal stability of lipase was also increased upon entrapment. Double immobilization was also investigated to fine-tune the sol-gel immobilization approach. Tomin et al., [2011] performed a systematic study of the sol-gel immobilization of Celite-supported lipase using ternary silane precursor systems of various alkyltriethoxysilanes (alkylTEOS), phenyltriethoxysilane (PhTEOS) and tetraethoxysilane (TEOS) to improve the diffusion of the substrates or products to and from the enzyme and eventually increase the reaction rate.

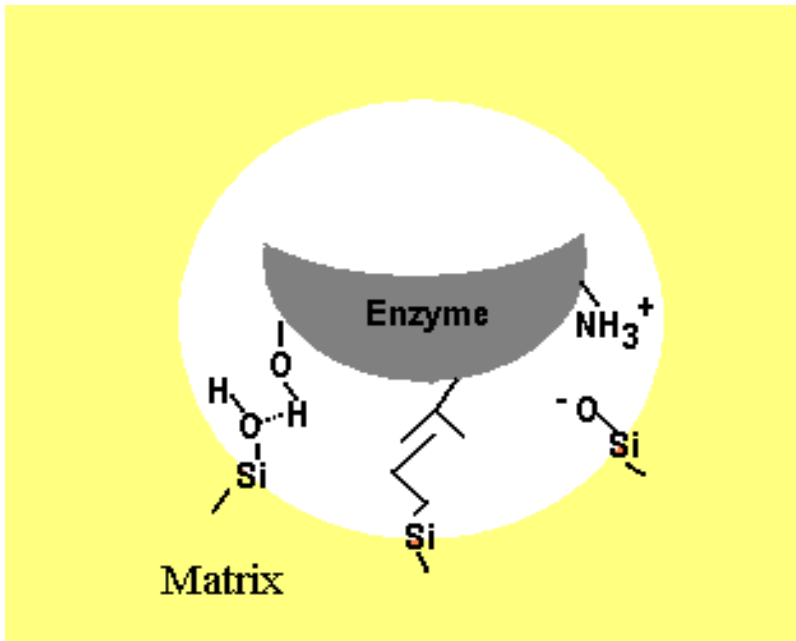

Fig. 2.4 Schematic view of noncovalent interactions between the gel matrix and the lipase [Reetz, 1997]

2.5 Biodiesel

Depletion of fossil fuels and global warming promoted the intensive investigation for sustainable biofuels instead of fossil fuels. One of the alternative energy sources is biodiesel. It has attracted attention nowadays due to its renewability, biodegradability and environmentally friendliness since it is non-toxic and clean burning. Biodiesel reduces the emissions of carcinogenic compounds around 85% compared to petrodiesel. B100, pure form of biodiesel, can be blended with petrodiesel to increase its flash point for safer fuel usage, handling and storage. This can be used in existing engines, vehicles and infrastructure with no further modification of the equipment. However, biodiesel has its disadvantages such as high viscosity, lower energy content, high cloud and pour point, high nitrogen oxide emission, lower engine speed and power, injector cooking, high price and engine corrosion [Demibras, 2009].

Biodiesel can be produced from oils, fat, lard and tallow. Raw materials, which are widely available and with appropriated physicochemical characteristics, enable cost reduction of biodiesel production since the feedstock cost represents 70-88% of the final price of biodiesel [Dabdoub et al., 2009]. Natural oils and fats are triglyceride esters of glycerol and three fatty acids molecules. The feedstock which could be mixtures of fatty acids (FFA) and

triglycerides (TAG) need to be chemically converted to fatty acid alkyl esters (FAAE) to be useful as biodiesel fuel [Meher et al., 2006]. Transesterification and esterification of plant oils and animal fats is the method widely used for biodiesel production. Sustainability of various feedstock materials, catalytic methods, process parameter optimization and improvement of product quality has been investigated to make the biodiesel production economically-viable and environmentally friendly [Sharma et al., 2008].

2.5.1 Biodiesel Production Methods

The common and most cost-efficient method for biodiesel production is the alcoholysis (transesterification) of triglyceride oil with alcohol in the presence of a catalyst which yields monoalkyl esters and glycerol (Fig. 2.5). Three-step reaction occurs in which triglycerides are converted to diglycerides, diglycerides to monoglycerides and finally monoglycerides to glycerol. Monoalkyl ester of fatty acid is produced in each corresponding steps. Three moles of alcohol are needed for the conversion of one mole of triglyceride into biodiesel. However a higher amount of alcohol is usually added to drive the reaction in the forward direction [Leca et al., 2010].

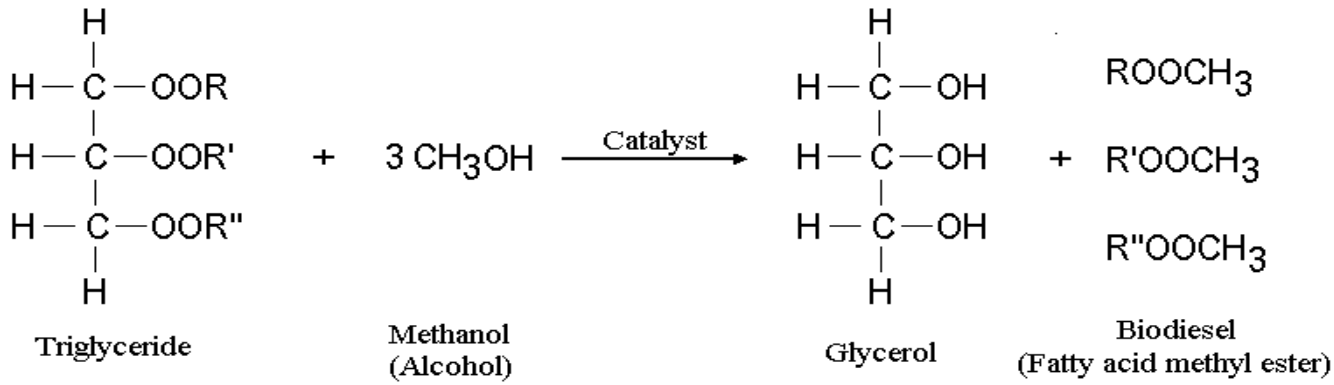

Fig. 2.5 Transesterification process where R, R' and R" represent alkyl chains

Homogeneous and heterogeneous catalysts (acid and base), and enzyme catalysts are being used to catalyze the transesterification reaction. Alkaline transesterification is most often used in industry today. It has faster rates and greater yields than the acid-catalyzed process. Sodium hydroxide is the preferred catalyst due to its lower price and higher biodiesel yields. When feedstock material has high free fatty acids or water content, it will be pretreated with acidic catalyst in order to esterify FFA [Zhang et al., 2003]. Pretreatment is necessary in order to reduce the formation of soap during reaction and ease the separation of biodiesel and glycerol.In the presence of water higher than 0.3%, there is a possibility of alkaline-catalyst consumption that results to low reaction yield. Wastewater generation and treatment pose a severe problem both from an energy consuming and environmental point of view for the chemical transestification method. These problems with soup formation and product separation have prompted research with heterogeneous non-enzymatic catalysts like zeolites, alkaline earth metal oxides, and solid acids like zirconia and alumina and sulfated tin oxide. Heterogeneous catalysts have the advantages of easier recovery and usability on substrates containing higher concentrations of FFA compared to homogeneous catalysts. However heterogeneous catalysts are costly and energy intensive due to high reaction temperatures and high alcohol to substrate molar ratios [Lam et al., 2010].

The problems associated with the use of chemical catalysts can be minimized or even eliminated by the application of enzymes as catalysts for biodiesel production. Enzymes such as lipases offer a biological route for biodiesel synthesis with environmental and economic advantages. The enzyme-catalyzed transesterification is more suitable for use on FFA-rich feedstock materials since FFA is directly esterified enzymatically into fatty acid methyl esters (FAME). Thus, lipase can be used with broader substrate range due to the ability of the enzyme to esterify the feedstock in one-step process. Enzyme-catalyzed reactions eliminate the treatment cost associated with recovery of chemical catalysts since the enzyme can be immobilized for easier separation, reuse and stability improvement. Enzymes are also biodegradable and environmentally acceptable. The enzyme catalysis is considered for biodiesel production due to its high product purity and greener technology. However, the product obtained from enzymatic processes is expensive due to the high cost of enzymes. In addition, enzyme activity is inhibited by the short chain alcohols used as acyl acceptor in the transesterification process. The development of new techniques to overcome the constraints related with enzyme catalysis is now being address by researchers and needs further investigation to have a cost-effective enzymatic biodiesel production. Table 2.2 summarizes the advantages and disadvantages of the various catalysts used for biodiesel production.

Table 2.2 Advantages and disadvantages of various catalysts used in biodiesel production [Guldhe et al., 2015]

Catalyst Type	Advantage	Disadvantage
Homogeneous Alkaline	High yield, low cost, fast reaction rate, medium energy requirement	Saponification of FFA, generation of wastewater, difficult catalyst recovery, purification of products required
Homogeneous Acid	Medium yield, conversion of FFA to biodiesel, low cost, medium reaction rate	Generation of wastewater, difficult catalyst recovery, purification of product required, corrosion of equipments, high energy requirement
Heterogeneous Alkaline	High yield, medium cost, reusability, fast reaction rate, can be used in continuous process	High energy requirement, tedious catalyst preparation, catalyst leaching, saponification of FFA
Heterogeneous Acid	High yield, reusability, medium cost, fast reaction rate, conversion of FFA to biodiesel, can be used in continuous process	High energy requirement, tedious catalysts preparation, catalyst leaching, corrosion of equipments
Enzyme (Immobilized Lipase)	High yield, conversion of FFA to biodiesel, low energy requirement, high product purity, reusability, no wastewater generation, can be used in continuous process	Inhibition by alcohols, high cost

2.5.2 Factors affecting biodiesel production

The efficiency and yield of biodiesel synthesis is influenced by various factors such as the nature and properties of the catalyst, biodiesel substrates, acyl acceptors, operating conditions and bioreactor design. If the enzymatic catalysis is applied, the nature and properties of the enzyme catalyst can also affect biodiesel production. Aside from that, the addition of acyl acceptors, the use of solvent, water content, enzyme pretreatment and immobilization technique should be considered.

Acyl acceptor. For commercial production of biodiesel, the use of cheap and readily available acyl acceptor such as methanol and ethanol is required. The yield of biodiesel increases with the increase in the alcohol concentration up to a certain level depending on the particular operating conditions. An excess of alcohol is also required to shift the equilibrium to fatty acid alkyl esters (FAAE) formation. Methanol is commonly used for transesterification because of its reactivity, volatile nature and lower cost. However, lipases can be inactivated by methanol that affects their catalytic efficiency [Chen and Wu, 2003]. There are strategies to minimize methanol inhibition: (a) stepwise addition of alcohol; (b) use of solvents; (3) the use of alternative acyl acceptors such as longer chain

alcohols and alkyl esters; and (4) the application of methanol-tolerant lipase. The choice of alcohol can affect the lubricity of the biodiesel [Drown et al., 2001]. The longer chain alcohols have shown higher yields than methanol as lipases have higher affinity towards long-chain than short chain alcohols [Fukuda et al., 2001].

Watanabe et al., [2000] investigated a two-step methanol addition batch process and three-step methanol addition continuous process for methyl ester production from vegetable oil using immobilized *Candida antarctica* lipase. Conversions of 95% and 93% were obtained in the batch and continuous process respectively. Likewise, during batch and continuous biodiesel production using stepwise addition of methanol to waste oil, 96% and 92% yields were achieved for the batch and continuous process of an immobilized *Candida* sp 99-125 lipase respectively. The lipase retains full activity after 20 days of continuous operation [Nie et al., 2006].

Addition of Solvent. Enzymatic catalysis can be carried out in the presence or absence of solvent. Solvent reduces viscosity and helps in promoting proper mass transfer of the substrate and product. The solvent provides better interaction of substrates with the active site of the enzyme since solvents increase the solubility of oils and alcohol, but there will be an additional cost for

its removal after the reaction. Hexane, n-heptane, petroleum ether and *tert*-butanol are the commonly used solvents for transesterification reaction [Ghaly et al., 2010]. Ranganathan et al. [2008] investigated the use of *tert*-butanol as a solvent for biodiesel production from cotton seed oil using Novozyme® 435 for 24 hours at 55°C and obtained a yield of 97%. About 95% of the lipase activity retains after 500 hours of continuous operation. Due to the toxicity and flammability of some solvents, they have a detrimental effect on the environment so that their application must be in minimal.

Water Content. For lipase-catalyzed reactions, water plays a vital role and has strong influence on the catalytic activity and stability of the lipase [Lu et al., 2009]. Lipase activation by water involves conformational changes of the enzyme which is dependent on the availability of an oil-water interface [Panalotov and Verger, 2000]. Water content in the reaction can be determined by either water activity (a_w) or as weight percentage of feedstock oil. The control of water content in the reaction mixture is necessary since lipase requires a minimum amount of water to maintain its active conformation. However, an excess of water may promote the competing hydrolysis reaction of the substrate and generate diffusion limitations thereby causing a decrease of transesterification yields. Pandey [2009] studied the used of lipase from *Chromobacterium viscosum* in the alcoholysis of jatropha oil with 10% enzyme

loading. The enzyme was immobilized on Celite 545 and 71% yield was obtained. Adding 0.5% water to the immobilized enzyme increases the yield to about 92%. For Candida sp 99-125 lipase the optimum water amount required for higher biodiesel yields was 10-20% based on the oil weight [Lu et al., 2009, Nie et al., 2006 and Tan et al., 2006].

Reaction Temperature. Normally, transesterification is carried out below the boiling point of the alcohol used in the reaction to prevent alcohol vaporization. Higher reaction temperatures reduce oil viscosity, increase the reaction rates and shorten the reaction time [Freedman et al., 1986]. Compared to chemical reaction for transesterification, enzymatic catalysis is generally performed at lower temperature to prevent the loss of lipase activity. The conversion of transesterification is rarely influenced by temperature fluctuations if the reaction stays between 20–70^{o}C. However, optimum temperature determined for lipases used for biodiesel production ranges between 30–60^{o}C. This optimum temperature is dependent on enzyme stability, alcohol to oil molar ratio and the type of organic solvent used [Antczak et al., 2009]. An increase in temperature beyond the optimum promotes denaturation and loss of enzyme catalytic activity. Jeong and Park [2008] reported that the optimal temperature for Novozyme® 435 is 40^{o}C.

Feedstock Material. The choice of feedstock depends on the characteristics of used oils, process chemistry and economy of the process. One of the main constraints for biodiesel production is the cost of the feedstock. The high value of edible oils as food product makes the production of biodiesel very challenging economically. Animal-derived products and waste oil may also be suitable raw materials for biodiesel synthesis. Utilizing waste oils as feedstock solves its disposal problem thus minimizing the environmental concerns. Microalgae have recently emerged as one of the promising feedstocks for biodiesel synthesis. Easily available and sustainable feedstock materials should be considered to have an economically feasible and sustainable biodiesel production process.

Chapter 3

Simultaneous improvements in the activity and stability of *Candida antarctica* lipase B through multiple-site mutagenesis

3.1 Introduction

Enzymes are increasingly used in industrial applications because of their inherent stereo-selectivity and their potential for environmentally friendly processes. However, the performance of an enzyme may be affected by temperature, the organic solvent in the reaction medium, the substrate concentration and possible inhibitory products. In addition, enzymes are far more expensive than chemical catalysts. Hence, the development of enzymes that overcome these limitations would greatly increase their utilization in industrial processes.

There are various approaches used to optimize the functionality of an enzyme. These include changing the microenvironment of the enzyme or optimizing the enzyme itself via mutagenesis. Mutation technology can easily modify enzymes through random or site-directed mutagenesis. However, mutation technology focuses primarily on a single function of the enzyme, such as thermostability [Kim et al., 2010; Yu et al., 2014], organic solvent stability [Park et al., 2013], specificity change [Chen et al., 2014], activity enhancement [Hong et al., 2013], or the creation of new catalytic routes that are not native to the enzyme itself [Bornscheuer and Kazlauskas, 2004]. Engineering an enzyme with a higher stability often leads to a detrimental effect on its catalytic activity.

Therefore, it is essential to obtain an enzyme that possesses both robustness and higher activity.

Enzymes are dynamic molecules that exhibit oscillatory motions related to its catalytic function [Yon et al., 1998]. The dynamics and flexibility of each part of the enzyme contributes to the maintenance of its dimensional structure, functional activity and stability. Several studies have reported a correlation between the flexibility and the functionality of enzymes, such as that between activity and stability. The stability of an enzyme increases by rigidifying the flexible residues of the enzyme [Kim et al., 2010; Park et al., 2013]. On the other hand, enzyme activity increases upon increasing its flexibility [Hong et al., 2013]. However, making the enzyme more flexible for efficient catalytic activity has consequences on its stability, since the two properties tends exert an inverse effects [Arnold et al., 2001; Beadle and Shoichet, 2002].

The *Candida antarctica* lipase B (CALB) enzyme is the most widely studied lipase. It has various applications that promote its utilization in the pharmaceutical, chemical, food and biofuel industries. The enzyme consists of 317 amino acid residues with a classic α/β hydrolase folding structure, three disulfide bonds and a classic triad active site (Ser 105, Asp 187 and His 224). In this study, the functionality of the CALB enzyme was enhanced via multiple-

site mutagenesis. The flexibility of various regions of the CALB enzyme was modulated to obtain an enzyme that was stable in organic solvent while able to exert higher enzymatic activity.

3.2 Materials and Methods

3.2.1 Materials

The *Pichia pastoris* strain X-33, the *pPICZαA* plasmid and the QuikChange™ site-directed mutagenesis kit were purchased from Invitrogen (USA). The restriction enzymes XhoI, XbaI and DpnI were purchased from Enzynomics (South Korea). *Para*-nitrophenyl caprylate (*p*NPC) was purchased from Sigma-Aldrich (USA). All other chemicals were of analytical grade.

3.2.2 Cloning and transformation of CALB variants

The CALB gene was subcloned into the *pPICZαA* plasmid using XhoI and XbaI restriction enzymes. Site-directed mutagenesis of CALB was conducted using a modified QuikChange™ protocol. All primers (listed in Table 3.1) were synthesized at COSMO GENTECH (South Korea), and the

mutant genes were confirmed via sequencing at the same company. The *pPICZαA*-CALB recombinant vector was linearized via digestion with SacI and then transformed into the *Pichia pastoris* X-33 strain using the lithium chloride transformation method. The cells were spread on YPDS plates containing 500 to 1500 μg/mL of zeocin to select the multi-copied transformants.

Table 3.1 Mutation primers

Enzyme	Mutation Primer	Sequence
A8T	Forward	5’ CGGACCCTACCTTTTCGCAGCCCAAGTCGG 3’
	Reverse	3’ GGAAGGCCAAGCCTGGGATGGAAAAGCGTC 5’
A92E	Forward	5’ GCGCTCTACGAGGGTTCGGGCAACAACAAG 3’
	Reverse	3’ CGGTAGTGGCGCGAGATGCTCCCAAGCCCG 5’
N97Q	Forward	5’ CGGGCAAACCAAAGCTTCCCGTGCTTACC 3’
	Reverse	3’ GATGCGACCAAGCCCGTTGGTTTTCGAAGG 5’
V139E	Forward	5’ GGCACCGAACTCGCCGGCCCTCTC 3’
	Reverse	3’ CTGATGCTTCCGTGGTTCGAGCGG 5’
A151D	Forward	5’ GCGGTTAGTGACCCCTCCGTATGGCAGCAA 3’
	Reverse	3’ CTACGTGAGCGCCAATCACTGGGGAGGCATA 5’
T245S	Forward	5’ GCGCTCCACCTCGGGCCAGGCTCGTAG 3’
	Reverse	3’ CTAGGCGGGACGCGAGGTGGAGCCCGGTC 5’
I255E	Forward	5’ CTATGGCGAGACGGACTGCAACCCTC 3’
	Reverse	3’ CATCACGTCTGATACCGCTCTGCCTG 5’

3.2.3 CALB expression and purification

Positive transformations were inoculated with 25 mL of buffered glycerol-complex medium (BMGY; 1% yeast extract, 2% peptone, 100 mM potassium phosphate with pH 6.0, 1.34% YNB, $4x10^{-5}$% biotin, and 1% glycerol) in a 50-mL baffled flask. The cells were incubated at 29°C and maintained on a shaking incubator at 200 rpm until the culture reached an OD_{600} = 2-6. Then, the cells were harvested, and the cell pellet (OD_{600} approximately 1.0) was inoculated in 100 mL of buffered ethanol-complex medium (BMMY; identical to BMGY except for the use of 0.5% methanol instead of 1% glycerol). Methanol was added each day to maintain the concentration at 0.5%, and the temperature was maintained at 29°C. After 5 days, the cell-free medium was obtained by centrifugation (3500 rpm, 30 min, 4°C).

The supernatant containing the enzyme was concentrated using Amicon Ultra (Ireland) with a 30-kDa cut-off membrane. Cation exchange chromatography using an XK 16/20 column (Amersham Bioscience) filled with 7 mL source 15S resin was used for purification, according to the method of Trodler et al., [2008].

3.2.4 Activity assay and stability measurements

The activity of CALB was measured through the hydrolysis of *para*-nitrophenyl caprylate (*p*NPC). Purified CALB solution (20 µL with 75 µg/mL enzyme) was mixed with 1 mL substrate mixture (substrate mixture contained: 1 mL of 33.3 mM *p*PNC in acetronitrile and 9 mL phosphate buffer (50 mM, pH 7.0) with 0.2 mL of Triton-X 100). The absorbance of the *para*-nitrophenol product was read at 405 nm and 30°C using a Cary 50 UV-spectrophotometer (Varian, USA). The molar absorption of the *p*-NP was determined as 9.02 $mM^{-1}cm^{-1}$, and 1 unit of CALB was defined as the quantity of 1 µM *para*-nitrophenol released in 1 minute. Protein concentration was measured according to Bradford [Bradford, 1976] using bovine serum albumin (BSA) as a standard.

The organic solvent stability was determined by incubating CALB in 40% (v/v) alcohol for 24 hours at 30°C. The residual activity of CALB after incubation was detected according to the method described above. The residual activities were expressed relative to the original activity. All experimental data are represented as the average of three measurements.

3.2.5 *In silico* mutagenesis and flexibility analysis

The crystal structure of CALB (PBB code: 1TCA) was used for the investigation. For the flexibility analysis of CALB, the B-factor, the overall RMSD value and the disorder profile of 1TCA and of mutated CALB were used. Glutamic acid (E) and aspartic acid (D) were selected to mutate one edge of the α-helix and a near loop region to increase flexibility, as these amino acids have a high B-factor [Radivojac et al., 2004; Bhasharan and Ponnuswamy, 1988]. The B-factor and the overall root-mean-square deviation (RMSD) values were also considered to determine the rigidity of the mutated site. Predicting protein B-factor profiles (BFPred) server (http://einstein.cs.iastate.edu/bfpred/index.html) was used to analyze the normalized B-factor values. The overall RMSD values were predicted using the TLS Motion Determination server [Painter and Merritt, 2006] using the mutants generated at the What IF server [Vriend, 1990]. The solvent-accessible surface area was calculated using the GetArea (http://curie.utmb.edu/getarea.html) server.

3.3 Results and Discussion

3.3.1 Selection of mutation sites

To integrate the activity enhancement and the stability in an organic solvent for CALB, various studies were consolidated. We previously studied the activity enhancement of CALB due to flexibility modulation of the helix region surrounding the active site. Two mutants, V139E and I255E, showed an enhancement in specific activity with respect to wild type [Hong et al., 2013]. However, in general, the enzyme activity decreased significantly if it was used with an organic solvent [Klibanov, 1997; Broos et al., 1995]. The decrease in enzyme flexibility observed in organic solvents is thought to be the reason behind its corresponding decline in activity. On the other hand, four solvent-affecting sites (residues: A8, A92, N97, and T245) have been identified in CALB. Rigidifying these solvent-affecting sites may stabilize CALB in a hydrophilic organic solvent [Park et al., 2013], but the induced rigidity could lower its catalytic efficiency. Because activity and stability enhancements were previously considered separately and these two properties showed an inverse relationship, a suitable mutation site combination should be determined such that CALB obtain both stability and enhanced catalytic activity. Table 3.2,

shows the mutation sites from our previous studies with their corresponding location and exposure ratio (%) based on the solvent accessible surface area.

Aside from the computational design developed in our previous studies, the hydrophobicity profile of CALB was predicted using TopPred II [Von Heijne 1992; Claros and Von Heijne, 1994] in this study. The result shows that helices α5 (residues 139-146), α8 (residues 212-216) and α10 (residues 268-287) which surrounds the entrance to the active site of CALB are hydrophobic. These helices are also positioned towards the hydrophobic region, as predicted from the Orientation of Proteins in Membranes (OPM) server [Lomize et al., 2006]. This orientation is important because CALB is classified as a lipase that displays catalytic activity at water-lipid interfaces. In addition, CALB has a higher specific activity towards hydrophobic triglyceride interfaces than towards soluble esters [Martinelle et al., 1995]. Based on this orientation, CALB was divided into two regions (*e.g.,* the substrate-binding region and the hydrophilic solvent-affecting region), as shown in Fig. 3.1.

Table 3.2 Location of mutation sites and the exposure ratio of the residue

Residue No.	Amino Acid		SASA[a](%)	Secondary structure	Relative activity[b]	Reference
	Original	Mutant				
139	V	E	48.4	Edge of α-helix	4.30	[5]
151	A	D	1.0	Loop	-	
255	I	E	98.0	Edge of α-helix	3.50	
8	A	T	100.0	Loop	0.77	[6]
92	A	E	77.8	Within α-helix	1.30	
97	N	Q	69.6	Loop	0.97	
245	T	S	65.1	Loop	1.16	

[a] SASA: Solvent accessible surface area reported in exposure ratio (%).

[b] Relative activity is calculated with respect to the wild type.

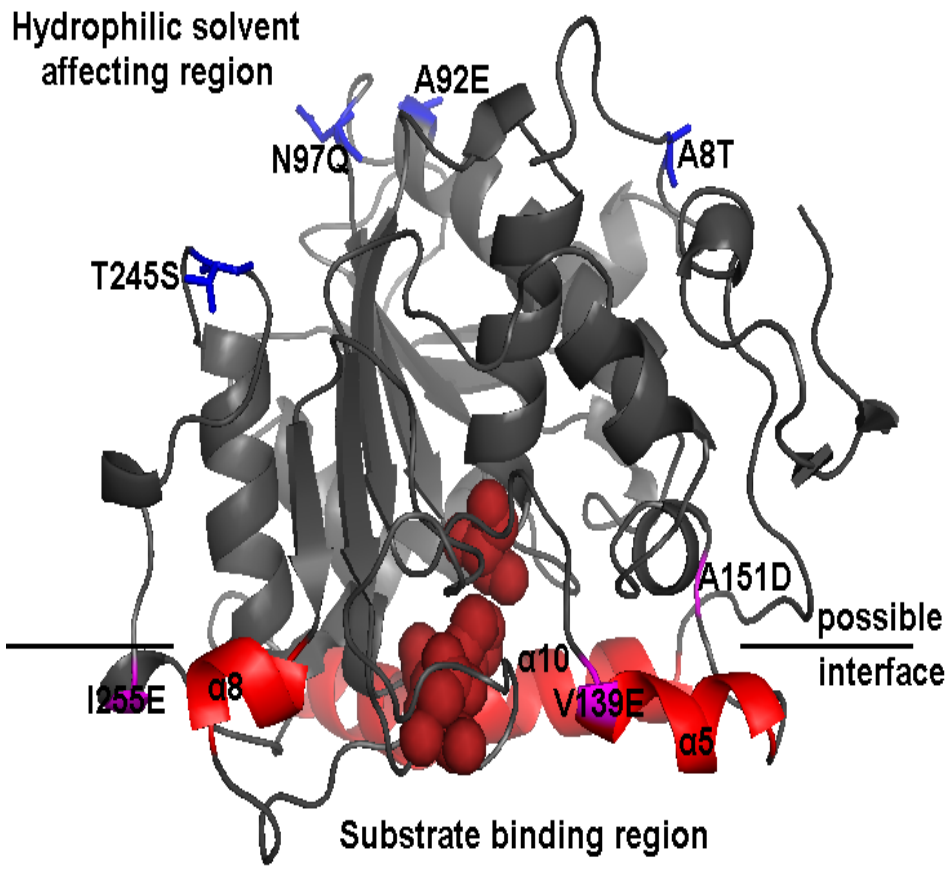

Fig. 3.1 Orientation of *Candida antarctica* lipase B [PDB ID: 1TCA] showing the two different regions, active site residues (in red spheres), mutation residues for activity enhancement (magenta) and mutation residues for stability enhancement (blue)

In the substrate-binding region, flexibility is modulated to increase the activity of the enzyme. It is expected that upon increasing the flexibility in the substrate-binding region, the activity of CALB will be enhanced and will lead to a greater flexibility for catalysis. The rigidity of the hydrophilic solvent-affecting region is also necessary to stabilize the enzyme in organic solvents. Each CALB regions was first optimized for its respective functionality (enhanced activity or enhanced stability) before integrating the mutation sites to obtain an entirely optimized enzyme.

3.3.2 Activity enhancement of the substrate-binding region

Flexibility of an enzyme is required in its folded state to accomplish its function, such as substrate binding, activity modulation and macromolecular interactions. Flexibility on the substrate-binding region of CALB is necessary to increase its catalytic efficiency. For CALB, it is thought that the short α5 helix may act as a lid that blocks the entrance to the active site. However, because CALB lacks the interfacial activation, it has been suggested that α5 more likely plays a role in the binding of the protein to a hydrophobic substrate interface [Martinelle et al., 1995].

Multiple-site mutagenesis was carried out to increase the activity of CALB. All mutants exhibited an increase in activity compared with the wild type (Table 3.3). This indicates that modulating the flexibility within the substrate-binding region enhances the activity of CALB. Double mutant V139E,A151D exhibited an increase in activity compared with its corresponding single mutants. However, the increase in the relative activity of the double mutant V139E,A151D was only approximately 15% compared with the single mutants; thus, further experiments utilizing the V139E and A151D single mutants were performed. Further investigations of the single mutants will pinpoint which mutant in the substrate-binding region exerts the greatest effect on the activity of the enzyme.

Table 3.3 Catalytic activity upon flexibility modulation on the substrate-binding region

Sample	Specific Activity (U/mg)	Relative Activity
Wild Type (WT)	1.36	1.0
V139E	5.24	3.9
A151D	5.92	4.4
I255E	4.40	3.2
V139E,A151D	6.21	4.6
V139E,I255E	5.71	4.2
A151D,I255E	3.81	2.8

The B-factor, which is commonly used to represent flexibility, was predicted using the BFPred server to support the experimental results. The B-factor indicates the degree of elastic scattering caused by positional disorder or the thermal motion of an atom [Parthanasarathy and Murthy, 2000]. Residues with higher B-factors are more flexible. Based on Table 3.4, local flexibility was increased upon the introducing E or D to the selected mutation sites. The single mutants V139E, A151D and I255E all showed an increase in local flexibility based on their B-factor values. Double mutations, *e.g.,* V139E,A151D (at the edge of the α5 helix and its near loop region) and V139E,I255E (at the edges of two separate helices), also showed an increase in their B-factor values, which corresponds to enhanced local flexibility. Mutations along the α5 helix and its near loop region yielded an approximately 9% increase in relative activity compared with mutations at the edges of the two separate helices, V139E,I255E. On the other hand, the overall root-mean-square deviation (RMSD) remained unchanged after mutation, indicating that the mutants are identical in conformation to the wild type.

Table 3.4 Normalized B-factor values for the different positions on the substrate-binding region

Position	WT	V139E	A151D	I255E	V139E, A151D	V139E, I255E	A151D, I255E
139	-0.121	**-0.112**	-0.156	-0.141	-0.154	-0.163	-0.141
151	0.167	**0.254**	**0.276**	**0.249**	**0.236**	0.13	**0.249**
255	0.507	0.503	**0.525**	**0.615**	0.454	**0.562**	**0.615**
Overall RMSD	3.77	3.77	3.77	3.77	3.77	3.77	3.77

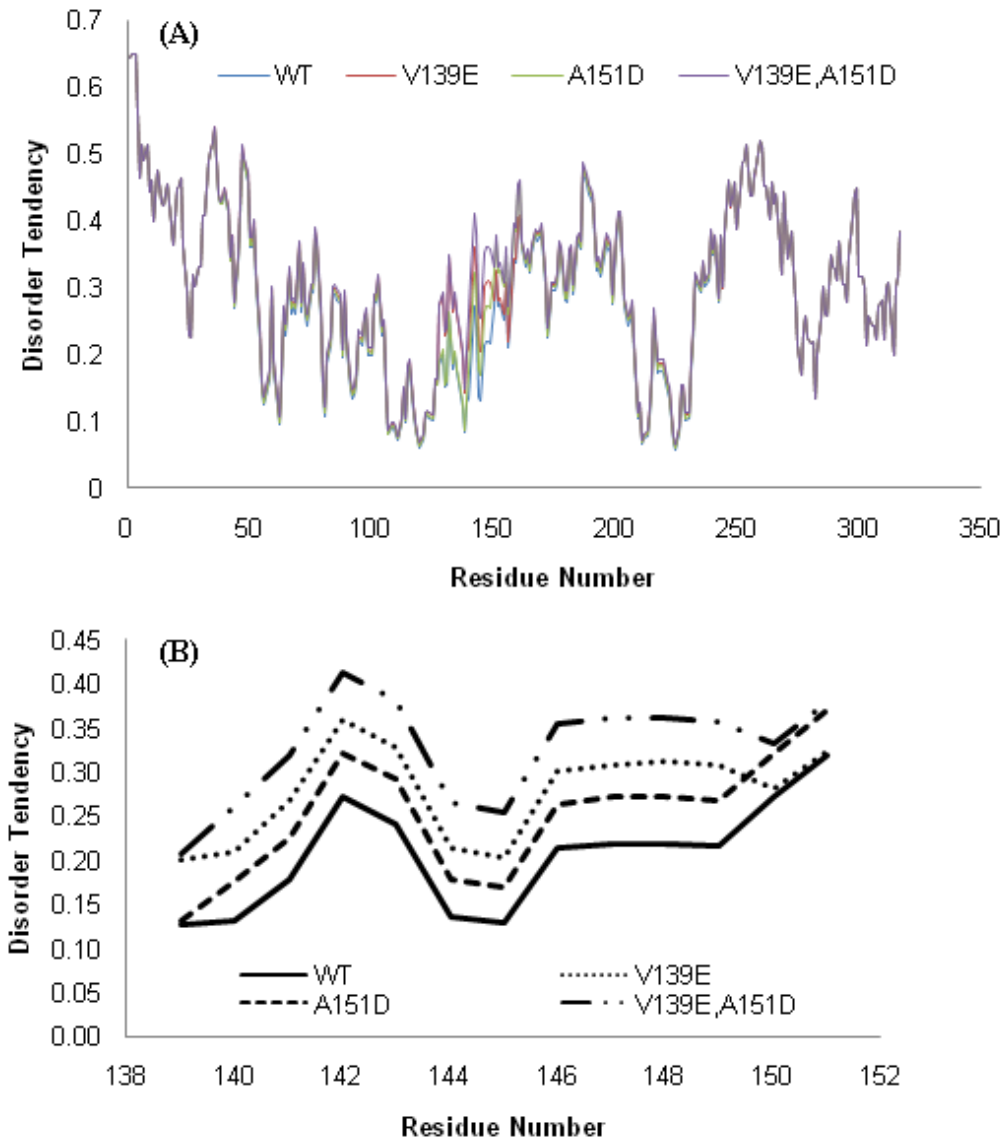

Fig. 3.2 Disorder tendency profile of CALB, (A) overall disorder tendency and (B) disorder tendency for residues 139-151

In addition to the B-factor values, prediction of intrinsically disordered proteins (IDP) was also performed to evaluate the flexibility of CALB. The IUPred server was used to predict IDP. This server utilizes a low-resolution energetic force field based on the pair-wise interacting residues observed in structures [Dosztányi et al., 2005]. The overall profile for the disorder tendency is shown in Fig. 2A. The disorder tendency profile deviations are more distinct within residues 139-151 as shown in Fig. 2B. Compared with the wild type, the disorder tendency deviated more in the α5 helix and the near loop region upon mutating with more flexible amino acids. The double mutation, V139E,A151D, exhibited the highest disorder tendency. This confirms that the α5 helix and its near loop region modulate the activity of CALB.

3.3.3 Stability enhancement of the hydrophilic solvent-affecting region

The stability of an enzyme is important for resisting denaturation and extending its shelf life for the synthesis of its products. To achieve a stable enzyme in organic solvents, mutations of the hydrophilic solvent-affecting region were performed. As shown in Fig. 3.3, CALB retained its activity after 24 hours when incubated in buffer solution only. When incubated in a 40% methanol solution, single mutants retained approximately 83-94% of their activity. Double mutations generally induced a residual activity greater than 90%

in 40% methanol, for which the double mutant A92E,T245S obtained the highest residual activity. The residual activity of the double mutant A92E,T245S induced differences of approximately 6% and 2% compared with the single mutants A92E and T245S, respectively. Thus, the single mutants A92E and T245S were used for further investigation.

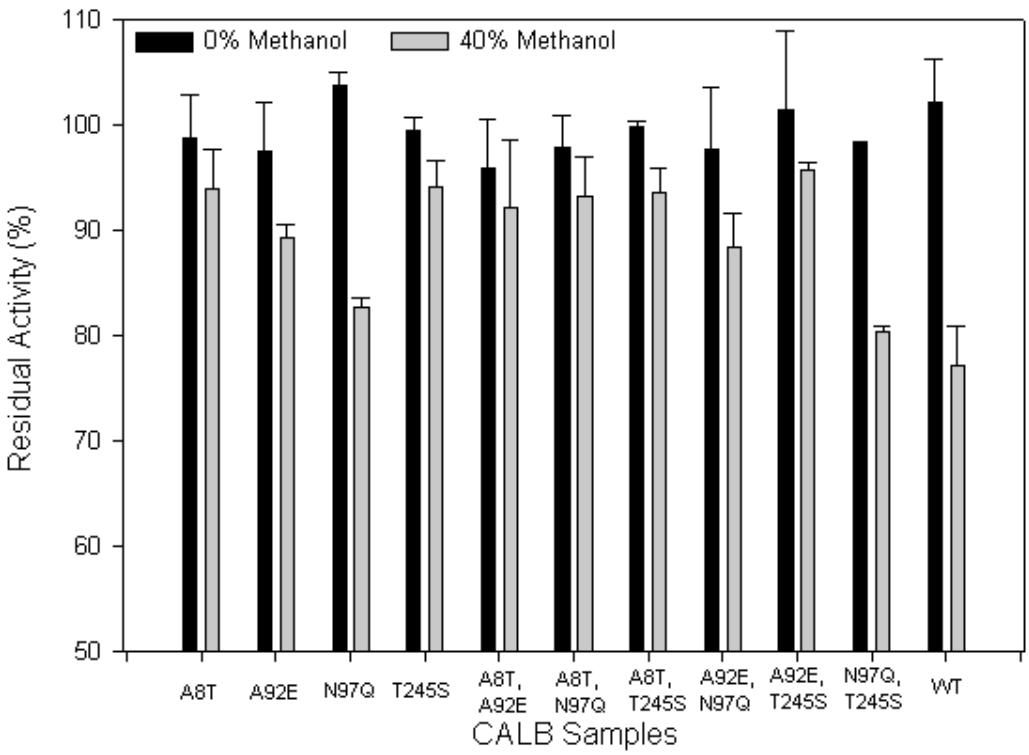

Fig. 3.3 Stability of the wild type and mutants of CALB on the hydrophilic solvent-affecting site using 75 µg/mL enzyme concentration

As presented in Tables 3.5 and 3.6, rigidity is expected upon a decrease in the B-factor value and the overall RMSD value of the enzyme. Rigidifying the hydrophilic solvent-affecting region can stabilize the enzyme upon incubation in organic solvents. Park *et al*. [2013] stabilized CALB through lowering the RMSD value of the methanol affecting sites. In the present study, it was shown that a double mutation further stabilizes CALB in an organic solvent. For the double mutant A92E,T245S, the B-factor value decreased at three mutation sites. This implies that the double mutant A92E,T245S promotes further rigidity of the hydrophilic solvent-affecting region compared with the single mutations. The overall RMSD for this mutant also decreased compared with the wild type.

Table 3.5 Normalized B-factor values for single mutants on the solvent-affecting region

Position	WT	A8T	A92E	N97Q	T245S
8	-0.152	**-0.155**	-0.113	**-0.164**	**-0.16**
92	0.335	0.335	**0.218**	0.356	**0.325**
97	0.497	0.533	0.58	**0.47**	0.538
245	1.292	1.324	1.378	1.351	1.321
Overall RMSD	3.77	3.76	3.76	3.77	3.77

Table 3.6 Normalized B-factor values for multiple-site mutagenesis on the solvent-affecting region

Position	A8T, A92E	A8T, N97Q	A8T, T245S	A92E, N97Q	A92E, T245S	N97Q, T245S
8	-0.129	**-0.183**	-0.146	**-0.2**	**-0.155**	**-0.208**
92	**0.212**	0.356	0.371	**0.295**	**0.212**	0.366
97	0.58	**0.47**	0.538	0.589	0.554	**0.477**
245	1.317	**1.277**	**1.272**	1.34	**1.269**	1.346
Overall RMSD	3.76	3.76	3.77	3.76	3.76	3.77

3.3.4 Integration of mutation sites for activity and stability enhancement

Reactions involving organic solvents require an enzyme with enhanced characteristics, such as increased catalytic activity and stability, to achieve economically feasible processes. From the results presented in this study, the mutations that enhance activity and the mutations that improve stability were integrated. As shown in Table 3.7, the A92E and T245S mutants exhibit less activity than to the V139E and A151D mutants. Combining the mutants, to incorporate both increased stability and activity for CALB, resulted in double mutation with activity higher than the single mutant with enhanced stability (*e.g.,* A92E and T245S). This supports the idea that making the substrate-binding region flexible enhances the catalytic activity of CALB.

Table 3.7 Catalytic activity for the selected mutants and the integration of mutation sites

Sample	Parameter considered	Specific Activity (U/mg)	Relative Activity
V139E	Enhanced activity	5.24	3.9
A151D	Enhanced activity	5.92	4.4
A92E	Enhanced stability	1.74	1.3
T245S	Enhanced stability	1.59	1.2
V139E,A92E	Integration of stability and activity	3.23	2.4
V139E,T245S	Integration of stability and activity	1.85	1.4
A151D,A92E	Integration of stability and activity	2.53	1.9
A151D,T245S	Integration of stability and activity	2.08	1.5

Fig. 3.4 shows the stability results when the CALB mutants were incubated in different types of alcohols. All of the mutants exhibited lower residual activity in methanol compared with ethanol and iso-propanol. Previous studies have shown that short chain alcohols inhibit enzyme activity [24,25]. The A151D mutant was the least stable enzyme in hydrophilic organic solvents. Double mutations V139E,A92E and V139E,T245S (with 65% and 64% residual activity in methanol, respectively) induced an improvement in stability compared with single mutations. Although the activity of double mutations was less than the activity of the single mutants with enhanced flexibility (*e.g.,* V139E and A151D), the lower activity was compensated by an increase in stability. Modulating the flexibility of the substrate-binding region and the hydrophilic solvent-affecting region can yield an enzyme with improved characteristics.

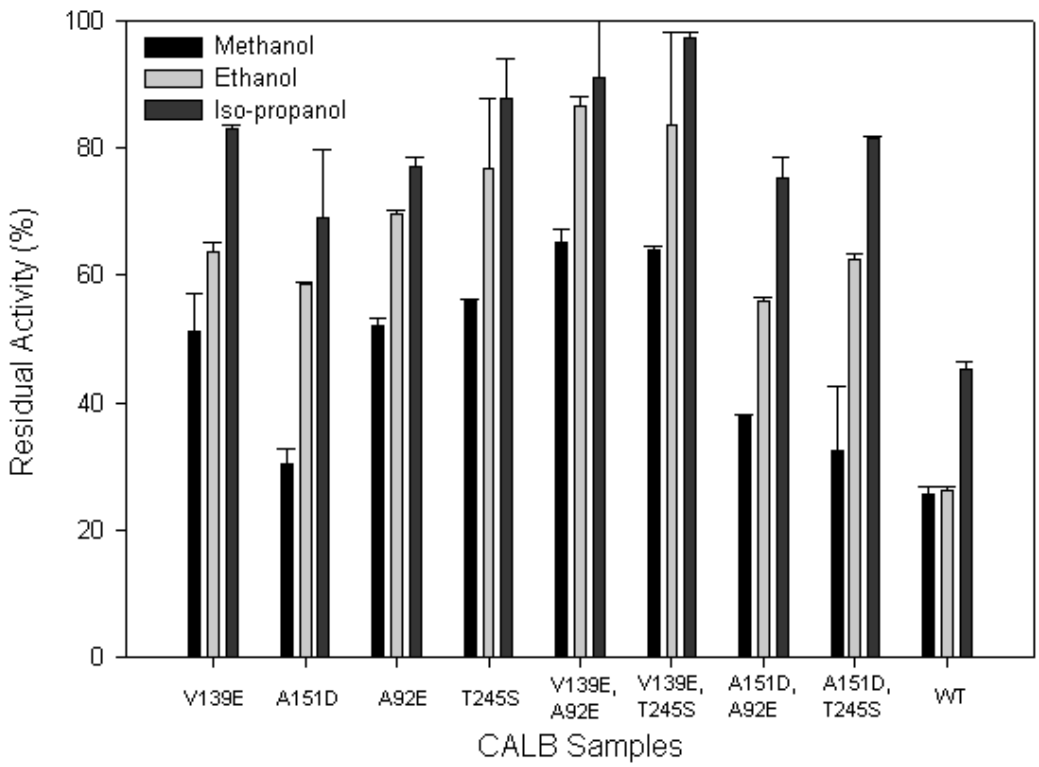

Fig. 3.4 Stability for the integrated mutation sites using different types of alcohol with 20 µg/mL enzyme concentration

3.3.5 Computational analysis between activity and stability

In order to analyze the importance of stability and activity for CALB, computational analysis is presented below. Assuming that the conversion of substrate S into product P is represented by the following reaction

$$E + S \leftrightarrow ES \underset{k_2}{\rightarrow} E + P$$

From Michaelis-Menten equation, the reaction rate is given as

$$v = \frac{(k_2 E)S}{K_m + S} = \frac{v_{max} S}{K_m + S} \qquad [1]$$

where E – active enzyme concentration

S – molar concentration of the substrate

v_{max} – maximum reaction rate

K_m – Michaelis-Menten constant or dissociation constant

k_2 – catalytic rate constant.

If the enzyme inactivation can be describe by a simple one-stage mechanism, such that

$$E_{native} \overset{k_d}{\rightarrow} E_{unfolded}$$

The first-order kinetic model is

$$-\frac{dE}{dt} = k_d E$$

$$ln\frac{E}{E_0} = -k_d t$$

$$\frac{E}{E_0} = exp(-k_d t) \qquad [2]$$

where k_d – inactivation rate constant

E_0 – active enzyme initial concentration.

For batch enzymatic reaction, a material balance on the reactor gives

$$-\frac{dS}{dt} = S_0 \frac{dX}{dt} = v(E, X) \qquad [3]$$

where X – substrate conversion

S_0 – initial substrate molar concentration

v(E,X) – reaction rate as a function of the enzyme concentration and conversion.

Substrate conversion for equimolar reaction is defined as

$$X = \frac{S_0 - S}{S_0} = \frac{P}{S_0}$$

$$S = S_0(1 - X) \qquad [4]$$

Case 1. Single substrate with no enzyme inactivation

Combing Equations 1, 3 and 4

$$S_0 \frac{dX}{dt} = \frac{v_{max} S_0 (1 - X)}{K_m + S_0(1 - X)}$$

$$\int_0^X \frac{K_m + S_0(1 - X)}{1 - X} dX = \int_0^t v_{max} dt$$

$$-ln(1 - X)K_m + S_0 X = v_{max} t$$

$$t = \frac{-ln(1-X)K_m + S_0 X}{v_{max}} \qquad [5]$$

Plotting X versus time, where v_{max} = 112.2 mM/hr, K_m = 0.879 mM and S_0 = 30mM from the actual experiments yields a curve as presented in Fig. 3.5. Increasing the value of the maximum reaction rate will speed up the conversion process. Sluggish reaction results to low v_{max} values.

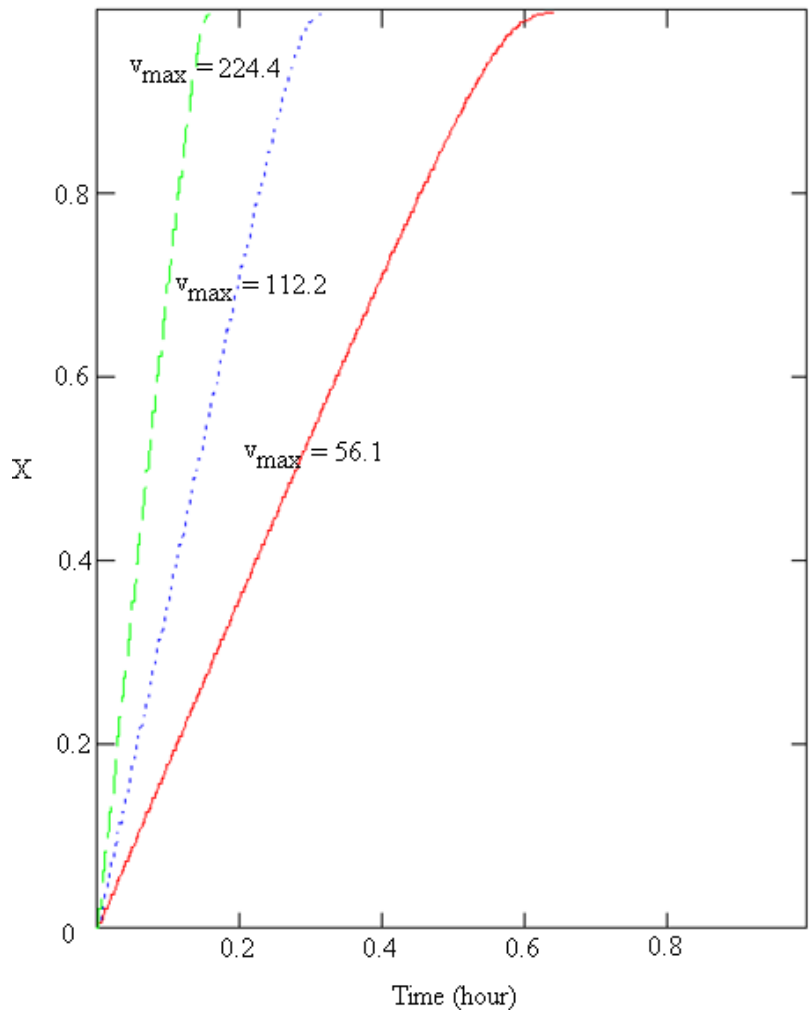

Fig. 3.5 Effect of changing the maximum reaction rate

Case 2. Enzyme inactivation in organic solvent

From Equation 2, $t = \frac{-ln\left(\frac{E}{E_0}\right)}{k_d}$ where E_0 = 30mM and k_d = 0.0085/hr from the actual experiments in methanol media [Park et al., 2013], the plot of E versus time is given in Fig. 3.6. From this figure, increasing the inactivation constant values leads to a more inactivated enzyme which in turn reduces its half-life values. In order to increase the life-span of the enzyme the inactivation constant should have a minimum value as much as possible. Increasing the inactivation constant twice will lower the half-life into half as shown in Table 3.8.

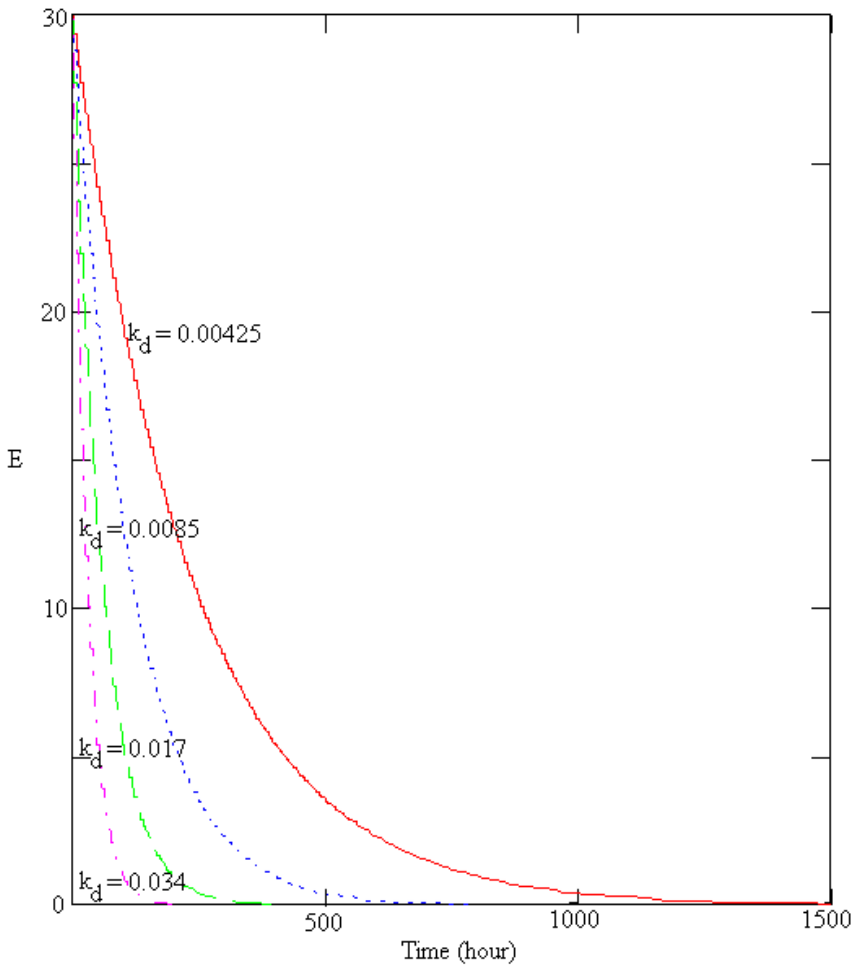

Fig. 3.6 Effect of different inactivation constants

Table 3.8 Inactivation constants and half-life values

k_d value (1/hr)	Half-life (hrs)
0.00425	163.09
0.0085	81.55
0.017	40.77
0.034	20.39

Case 3. Single substrate reaction with enzyme inactivation in organic media

From Equation 3 and substituting equations 1, 2 and 4

$$S_0 \frac{dX}{dt} = v(E, X)$$

$$\int_0^X \frac{dX}{v(E,X)} = \int_0^t \frac{dt}{S_0}$$

$$\int_0^X \frac{dX}{\frac{(k_2 E)S}{K_m + S}} = \int_0^t \frac{dt}{S_0}$$

$$\int_0^X \frac{dX}{\frac{k_2 E_0\, exp(-k_d t) S_0 (1-X)}{K_m + S_0(1-X)}} = \int_0^t \frac{dt}{S_0}$$

$$\int_0^X \frac{K_m + S_0(1-X)}{S_0(1-X)} dX = \int_0^t \frac{k_2 E_0\, exp(-k_d t)}{S_0} \mathrm{dt}$$

$$\int_0^X \frac{K_m + S_0(1-X)}{(1-X)} dX = \int_0^t k_2 E_0\, exp(-k_d t) \mathrm{dt}$$

$$-ln(1-X)K_m + S_0 X = \frac{1}{k_d} k_2 E_0 - \frac{1}{k_d} k_2 E_0 exp(-k_d t)$$

$$\frac{-ln(1-X)K_m + S_0 X}{\frac{k_2 E_0}{k_d}} = 1 - \ exp(-k_d t)$$

$$exp(-k_d t) = 1 + \frac{ln(1-X)K_m - S_0 X}{\frac{k_2 E_0}{k_d}}$$

$$t = -\frac{ln\left[1+\frac{ln(1-X)K_m - S_0 X}{\frac{v_{max}}{k_d}}\right]}{k_d} \qquad [6]$$

When the inactivation constant is within 0.00425~0.017 per hour, the rate of substrate conversion is almost unaffected. Doubling or lowering the k_d value by half will not greatly affect the conversion rate. However if the actual 0.0085/hr value of the inactivation constant is multiplied 100 times (new k_d = 0.85), then the amount of time to reach 100% conversion is changed from 0.322 hours to 0.375 hours as shown in Fig. 3.7. This is only about 14% reduction in time for complete conversion with a 100x reduction in k_d value.

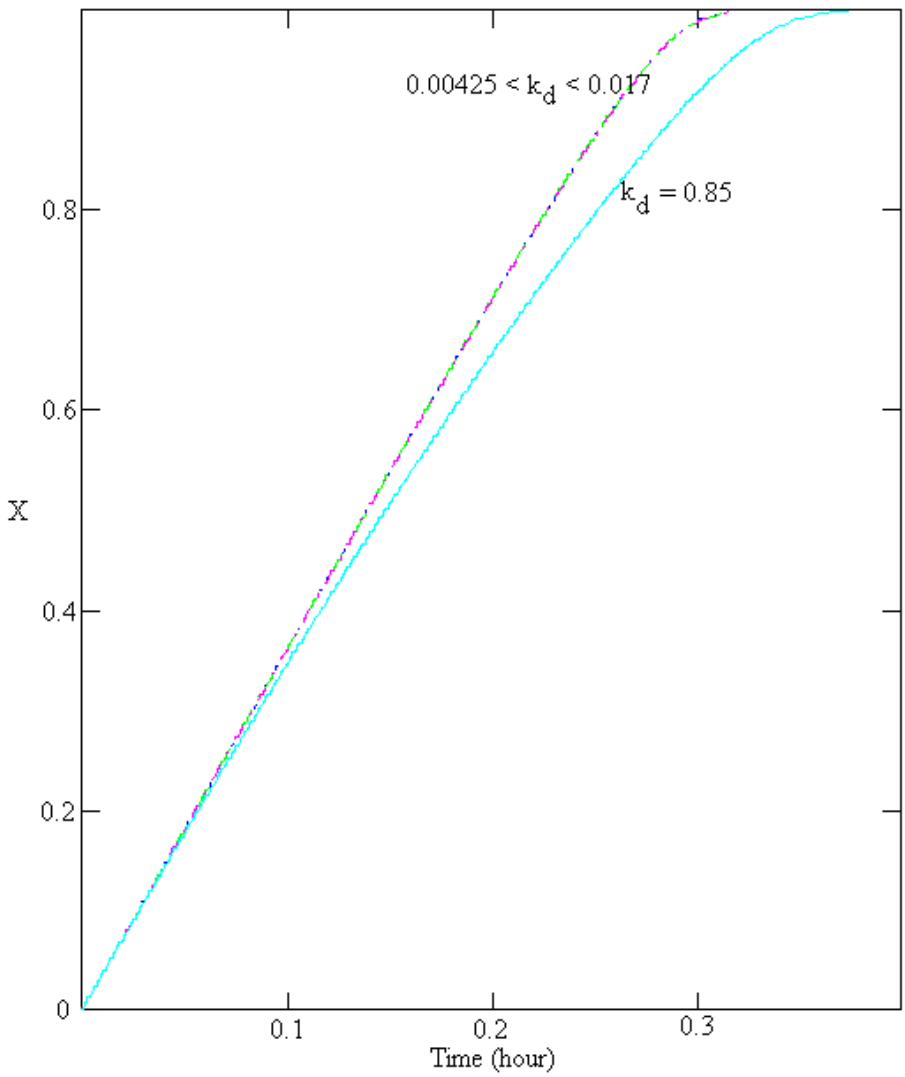

Fig. 3.7 Effect of inactivation constants at v_{max} = 112.2 mM/hr

Fig. 3.8 shows the effect of v_{max} values on the conversion rate. Increasing the v_{max} values will speed up the conversion. Low v_{max} values of the enzyme imply that the reaction will become sluggish. Comparing Case 1 (without enzyme inactivation) and Case 3 (with enzyme inactivation at k_d = 0.0085/hr), the time to reach 100% conversion is about 0.321 and 0.322 hours respectively for v_{max}= 112.2mM/hr. This means that the v_{max} value of the enzyme is the determinant for the reaction rates and not the k_d since Case 1 and Case 3 is less affected by the enzyme inactivation. If v_{max} is high then the effect of the enzyme inactivation is negligible, as also shown in Fig. 3.7 for inactivation constant within 0.00425~0.017 per hour and Table 3.9.

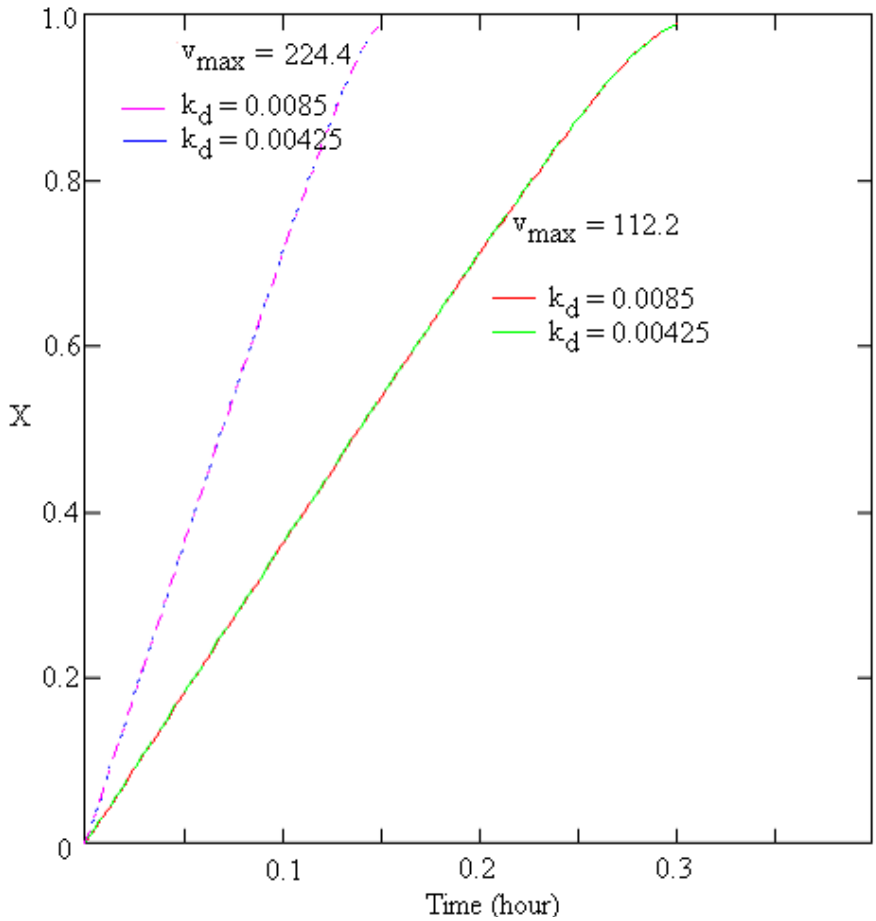

Fig. 3.8 Effects of v_{max} and k_d values

Table 3.9 Comparison between reactions with and without enzyme inactivation

v_{max} value	Time to reach 100% conversion		% difference in time
	Without inactivation	With inactivation	
56.1	0.642	0.644	0.31
112.2	0.321	0.322	0.31
224.4	0.161	0.161	0.00

Basing from the actual experimental values and through simulations, the activity of the enzyme is the more important factor to consider for higher product formation. Inactivation can be minimized if v_{max} is increased. There are two ways to increase v_{max}, one is to engineer the enzyme to have higher catalytic constant, k_2. The other one is to increase the active enzyme concentration, E.

3.4 Conclusion

The functionality of *Candida antarctica* lipase B (CALB) was improved by modulating the flexibility of its substrate-binding region and hydrophilic solvent-affecting region. Double mutations on each region further increased the enzyme's activity and stability. Integrating mutation sites to incorporate both the stability and flexibility of CALB is feasible, which can yield an enzyme with enhanced characteristics. The CALB V139E,A92E mutant, exhibited the highest stability in methanol and improved catalytic activity compared with the single A92E mutant. Hence, V139E,A92E can be applied to reactions involving organic solvents.

Chapter 4

Immobilization of *Candida antarctica* lipase B on the surface of a modified sol-gel matrix

4.1 Introduction

Enzyme immobilization has become a valuable means to stabilize the enzyme. Immobilizing enzyme can result in increase of thermal and operational stability if properly tuned, which allows their application in wider range of operating conditions, compared to the free enzymes. Different immobilization techniques have been applied to enzymes, including adsorption onto solid supports, cross-linking, covalent attachment and entrapment in polymers or inorganic matrices. Carrier-free enzyme immobilization by cross-linking offers advantages such as highly concentrated enzyme activity in the catalyst, high stability and the low production cost due to the exclusion of an additional carrier [Yu et al., 2006]. However, carrier-free enzyme immobilization systems are produced in fine powders which limit their suitability in industrial bioreactors due to separation problems and higher bed resistance. Carrier-bound enzyme immobilization facilitates ease of separation and catalyst reuse. The carrier will not only serve as the scaffold material for immobilization but it can also be a modifier that dictates the catalytic properties of the enzyme [Cao, 2003].

Sol-gel materials as carrier for enzyme immobilization exhibit valuable properties such as high surface to volume ratio, large surface area and porosity due to their nano or microporous structure [Pirozzzi et al., 2009]. Sol-gel immobilization allows high compositional and morphological flexibility by utilization of alkoxyde-type silane precursors holding one or two nonhydrolizable organic functional groups and various additives, to yield organic-inorganic hybrid matrices [Reetz and Jaeger, 2003]. Many additives or templates (e.g. polyethyleneglycol, glycerol, polyvinylalcohol, sugars, cyclodextrins) have been tested to improve the characteristics of the sol-gel material [Pierre, 2004]. As compared to organic type of supports, sol-gel materials exhibit higher mechanical strength and negligible degree of swelling.

Sol-gel encapsulation has been used for enzyme immobilization. Increased in thermal stability and activity appears to results from multipoint interactions such as hydrogen bonding as well as ionic and hydrophobic interactions. However, entrapment of enzyme in the sol-gel matrix will expose enzyme to some harsh conditions during the entrapment process since the solvent used, the enzymes and the acid or base catalyst are mixed together. Aside from that, accessibility of the entrapped enzymes might pose a problem for bulky type of substrates. Furthermore, as a result of their small molecular size, enzyme may slowly leak out from the matrix during continuous use.

Immobilization of enzyme by covalent binding on the solid carrier backbone overcomes these disadvantages.

Lipases (EC 3.1.1.3) are among the most widely employed biocatalysts due to their ability to catalyze various arrays of reactions, such as hydrolysis, esterification and transesterification [Wu et al., 2012; Sutili et al., 2013; Narwal and Gupta 2013]. Among them, *Candida antarctica* lipase B (CALB) exhibits high degree of substrate selectivity both with respect to regioselectivity and enantioselectivity. CALB also shows stability at high temperature and in organic solvents which made it one of the most frequently used enzyme for industrial application [Anderson et al., 1998]. CALB lacks the mobile lid that is commonly observed in most lipases but it is still considered as a typical lipase since it has higher activity in water-lipid interfaces and when attached to hydrophobic environment [Laszlo and Evans, 2007].

In this study, modified sol-gel matrix was utilized to immobilize CALB. Instead of encapsulating the enzyme into sol-gel matrix as was commonly done, covalent immobilization on the surface was investigated. Since sol-gel materials are flexible and can be easily modified, the free hydroxyl group on its surface was activated and consequently used for covalent immobilization.

4.2 Materials and Methods

4.2.1 Materials

Candida anatarctica lipase B (CALB) was purchased from Novozyme (Bagswaerd, Denmark). Methyltrimethoxysilane (TMOS), ethyltrimethoxysilane (ETMS) and 3-aminopropyltriethoxysilane (APTS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

4.2.2 Support preparation and activation

Sol-gel formation

Twelve milliliter of deionized water processed through Milli-DI® purification system was placed in a 50 mL Falcon tube. A 200 μL of 1 M sodium fluoride (NaF) as catalyst was added and the solution was shaken with a vortex mixer. Corresponding amounts of the precursors, tetramethylorthosilicate (TMOS) and ethyltrimethoxysilane (ETMS), were then added. The mixture was allowed to react overnight at 30^{o}C with rotational speed of 150 rpm. After

reaction, the sol-gel matrix was allowed to dry at 30^{o}C before it was ground to obtain the desired particle size.

Surface treatment of the sol-gel matrix

One gram of the previously prepared sol-gel powders were immersed into a 10 mL freshly prepared 5% (v/v) solution of 3-APTS in ethanol at 30^{o}C for 10 minutes then filtered and rinsed twice with 5 mL ethanol to remove unlinked 3-APTS. Alkylamine-derivatized sol-gel powders were dried before 10 mL of 1% glutaraldehyde (v/v in deionized water) was added and allowed to react for another 10 minutes. After reaction the powders were thoroughly washed with deionized water and dried. The treated powders were stored at 4^{o}C until further use.

4.2.3 Enzyme immobilization

Different volumes of CALB solution were mixed with 0.2 M phosphate buffer (pH 7) to have a 5, 7 and/or 10% (v/v) enzyme solution. One gram of the surface treated sol-gel powders were added to 10 mL of the enzyme solution. The free terminal aldehydic groups on the sol-gel matrix surfaces were subsequently cross-linked to amines on the enzyme through Schiff's base

formation. The immobilization was carried out at 30^{o}C for 24 hours under gentle stirring. After immobilization, immobilized enzyme matrix was wash thoroughly with phosphate buffer to remove unbound enzymes. The immobilized enzyme matrixes were dried in a vacuum and stored at 4^{o}C until further use. Schematic diagram for the surface treatment and enzyme immobilization methods is shown in Fig. 4.1.

Scanning electron microscope, AURIGA^{TM} (FIB-SEM), was used to obtain images of the sol-gel material and the enzyme immobilization system. The samples were coated with a thin layer of platinum in a customized sputter coating machine before the imaging process.

4.2.4 Enzyme activity assay

CALB activity was determined using 0.5% *p*-nitrophenyl palmitate in ethanol (99%) as a substrate. Free or immobilized CALB of known amounts were mixed with 2 mL substrate mixture (substrate mixture contains: 1 mL of 0.5% p-nitrophenyl palmitate in ethanol and 9 mL phosphate buffer (50 mM, pH 7.0) with 0.2 mL of Triton-X 100). After incubation for 5 minutes at 30^{o}C, the reaction was terminated by filtration. The absorbance of product *p*-nitrophenol was read at 405 nm using a UV-spectrophotometer. The molar

absorption of *p*-nitrophenol was determined to be 9.02 mM/cm and 1 unit of CALB was defined as the released quantity of 1μM *p*-nitrophenol in 1 minute.

4.2.5 Protein determination

Protein concentration was measured according to Bradford [1976] using bovine serum albumin (BSA) as a standard.

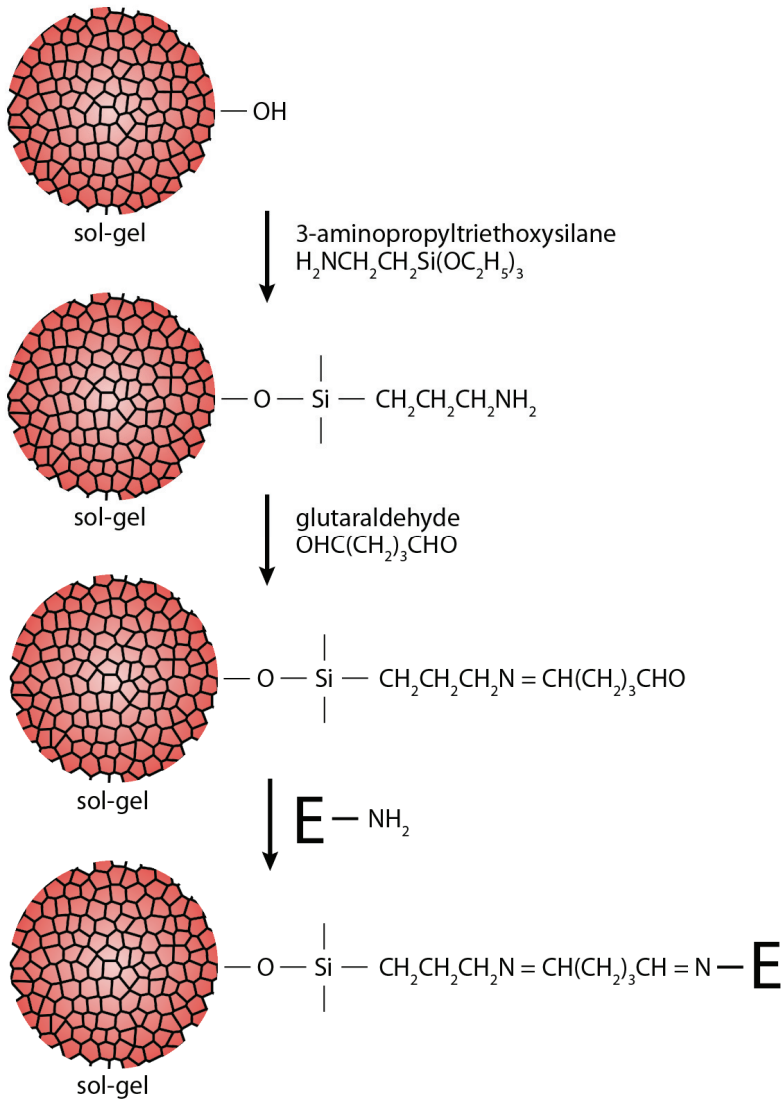

Fig. 4.1 Scheme for surface treatment of sol-gel matrix and immobilization of CALB on the matrix

4.2.6 Stability determination

Free and immobilized CALB were incubated in 0.2 M phosphate buffer (pH 7) at 60^{o}C for 24 hours for thermal stability determination. Periodically samples were withdrawn and their residual activities were analyzed. The operational stability of the immobilized CALB was also investigated in repeated batch reactions. After each hydrolysis reaction of *p*-nitrophenyl palmitate in the operational stability test, the enzyme immobilization system was thoroughly washed with phosphate buffer and subsequently used in the next reaction. The residual activity of the immobilized CALB every after reaction was detected by the method described above. Residual activities were expressed relative to the original activity assayed at room temperature.

4.3 Results and Discussion

This study extends the usability of sol-gel matrix as immobilization support for CALB. Modified sol-gel matrix material was used solely to covalently immobilized CALB. Since CALB is immobilized after the formation of sol-gel material then the harsh process condition of encapsulation can be

avoided. For covalent immobilization, CALB is located on the surface of the support thus its active site will be readily available for the substrate.

Sol-gel synthesis involves hydrolysis, condensation and polycondensation. Silica alkoxides ($Si(OR)_4$) are the most commonly used precursors for sol-gel formation, where R stands for an alkyl group. The hydrolysis reaction, through the addition of water, replaces the alkoxide groups (OR) with hydroxyl groups (OH). These hydroxyl groups after polycondensation of sol-gel materials were exploited in this study to bind the lipase covalently. The surface of the sol-gel matrix was activated by 3-APTS and glutaraldehyde was used to link the lipase to the activated support.

The active site of CALB is composed of a Ser-His-Asp catalytic triad. Based from the solvent accessible surface area (SASA) analysis, CALB has 9 lysine residues of which 3 are exposed and located at the opposite side of the active site (see Fig. 4.2). These lysine residues are perceived to react with the aldehyde groups through multipoint covalent binding with the support. Since the exposed lysine residues are located far from the active site, severe conformational changes that affect enzyme activity can be minimized.

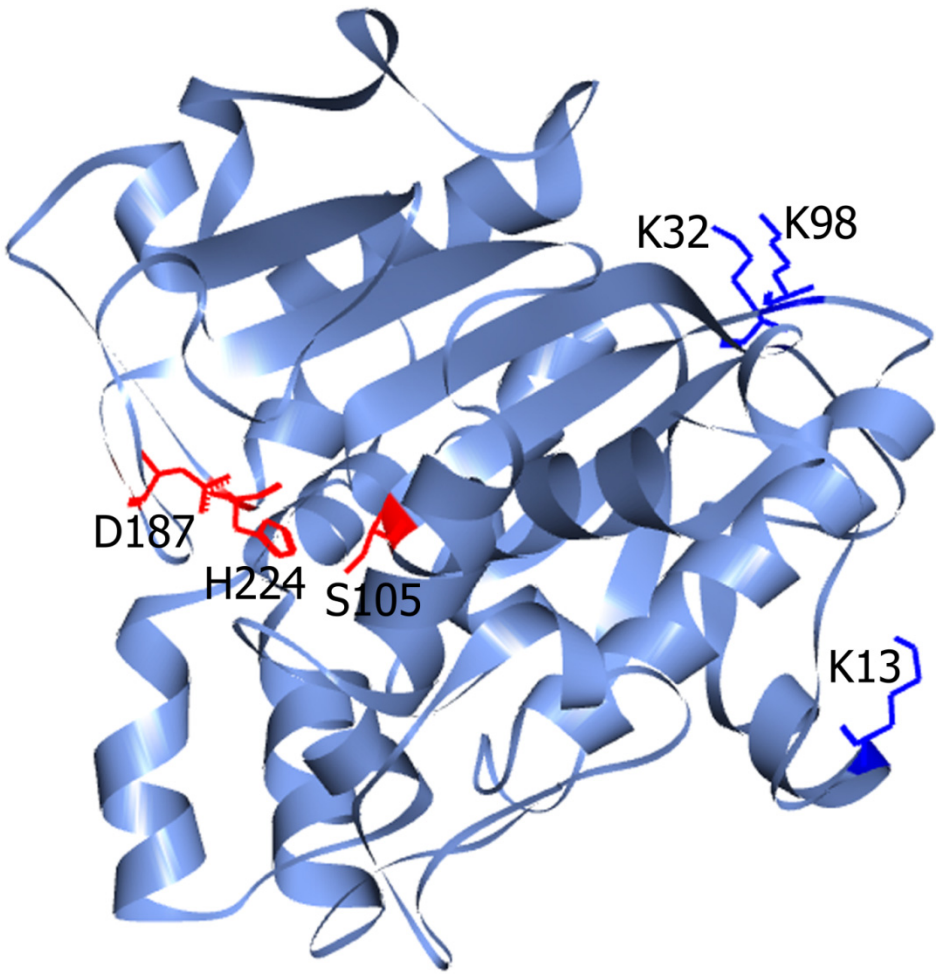

Fig. 4.2 Structure of *Candida antarctica* lipase B [PDB ID: 1TCA] showing active site residues (S105, D187 and H224) and exposed lysine residues (K13, K32 and K98)

4.3.1 Influence of sol-gel matrix size on attachment and enzyme activity

Particle size is important when assessing catalytic supports for enzyme-catalyzed synthesis and modification reactions. The effects of mass transfer on conversion and catalytic activity will be greater for macromolecular substrates relative to substrates with small molecules. The size of immobilization support can affect the attachment of the enzymes. Consequently, it determines the diffusion of substrates and products to and from the catalyst particularly if the enzymes are well packed or compressed on the support.

Table 4.1 Effect of solid support particle size on enzyme immobilization and activity

Particle size	% Attachment	Activity U/g beads	Activity U/g protein
–1000µm +500µm	39.34	4.91	12.10
–500µm +300µm	55.63	68.81	33.99
–300µm +100µm	63.90	115.41	29.57

In this study, the range of particle size is denoted with negative (–) and positive (+) signs. For example, particle size of (–1000µm +500µm) means that sol-gel matrix passed through 1000µm mesh size and retained on 500µm mesh size after thorough sieving. As shown in Table 4.1, an increase in protein attachment and enzyme activity results with a decrease in particle size. At enzyme loading of 611 µg/mL, percent attachment increasesabout 62% from a particle size of (–1000µm +500µm) to a particle size (–300µm +100 µm). Enzyme activity also increases 23 times. Higher activity can be attributed to the increase of enzyme load, increase in enzyme density of CALB molecules within the matrix and possible well distribution of the enzyme throughout the matrix when the particle size is smaller.

4.3.2 Sol-gel matrix composition

Methyltrimethoxysilane (TMOS) ($Si(OCH_3)_4$) as precursor is commonly used for sol-gel matrices. However, TMOS gave low enzyme activities [Reetz et al., 1995; Reetz et al., 1996] and is a hydrophilic compound which may not be suitable for CALB immobilization. Modified hydrophobic matrices prepared from alkyl-substituted organic silane precursors $RSi(OCH_3)_3$ improved the immobilization of lipases [Reetz et al., 1995; Reetz et al., 1996; Lee et al.,

2010]. In order to provide hydrophobic surface for CALB to attach, sol-gel compositions were varied in this study. Experiments were done with 611 μg/mL enzyme loading and a particle size of (–500μm +300μm).

The addition of ethyltrimethoxysilane (ETMS) to TMOS made the sol-gel matrix amphiphilic in nature. The amounts of ETMS dictate the hydrophobicity of the matrix. Based from the results presented in Table 4.2, TMOS to ETMS ratio of 1.18:2.56 gives the highest enzyme activity. Lipases are reported to have higher activity on hydrophobic environment, without the ETMS the sol-gel matrix is hydrophilic which could be the reason for lower activity. Higher amount of ETMS did not yield higher enzyme activity probably due to incomplete polycondensation of the matrix.

Table 4.2 Comparison of enzyme activity on different sol-gel precursor ratios

TMOS:ETMS (v/v)	% Attachment	Activity (U/g beads)	Activity (U/g protein)
1.18:5.12	50.00	28.37	9.12
1.18:2.56	55.63	68.81	33.99
1.18:0	50.00	29.62	9.52

4.3.3 Influence of enzyme loading on activity

The price of enzyme often hinders the process scale-up for enzyme-catalyzed processes thus enzyme loading capacity is an important parameter of immobilization. Different enzyme load were investigated with matrix particle size of (–500μm +300μm) and 1.18:2.56 TMOS/ETMS precursor ratio. Based from Table 4.3, higher enzyme loadings result to higher enzyme attachment and activity per amount of beads. Specific activity on the other hand decreases with increasing percent attachment. This can be attributed to high enzyme density on the matrix that may result to reduction of the porous effective diameter caused by spatial hindrance [Rodrigues et al., 2008].

Table 4.3 Effect of CALB loading on enzyme immobilization and activity

Enzyme Loading (μg/mL)	% Attachment	Activity U/g beads	Specific Activity U/g protein
611	55.63	68.81	33.99
883	56.00	101.27	20.47
1242	80.88	192.02	19.12

Silva et al., [2012] immobilized CALB on chitosan-based hydrogels and obtained enzyme activity of 190.86 U/g-support after drying using *p*-nitrophenyl butyrate as substrate. Their result is comparable to the one obtained from this study at 1242 μg/mL enzyme loading using a longer chain substrate, *p*-nitrophenyl palmitate. Immobilizing the enzyme on the surface can prevent the inaccessibility of the catalyst as evident in this present study. Lee et al., [2010] used sol-gel matrix to encapsulate oil pretreated CALB and obtained enzyme activity about 0.90 U/g-support. This result increased to about 75-200 times when the enzyme is covalently immobilized on the surface.

Scanning electron microscope (SEM) images are shown in Fig. 4.3. After thorough analysis of a large number of specimens in the samples, the presented images in this figure are chosen and believed to be a fair representation of the whole sample. The same magnifications are presented for the three figures to differentiate them. Fig. 4.3a shows the sol-gel matrix only without the activating agents. The sol-gel matrix is a porous type of material as reflected on the figure. Adding the activating and cross-linking agent reduces the porosity of this material (Fig. 4.3b). Further reduction in porosity and an increase in packing can be observed when the enzyme was added to the matrix. The reduction in porosity and a well packed surface suggest that the enzyme is attached on the surface of the support.

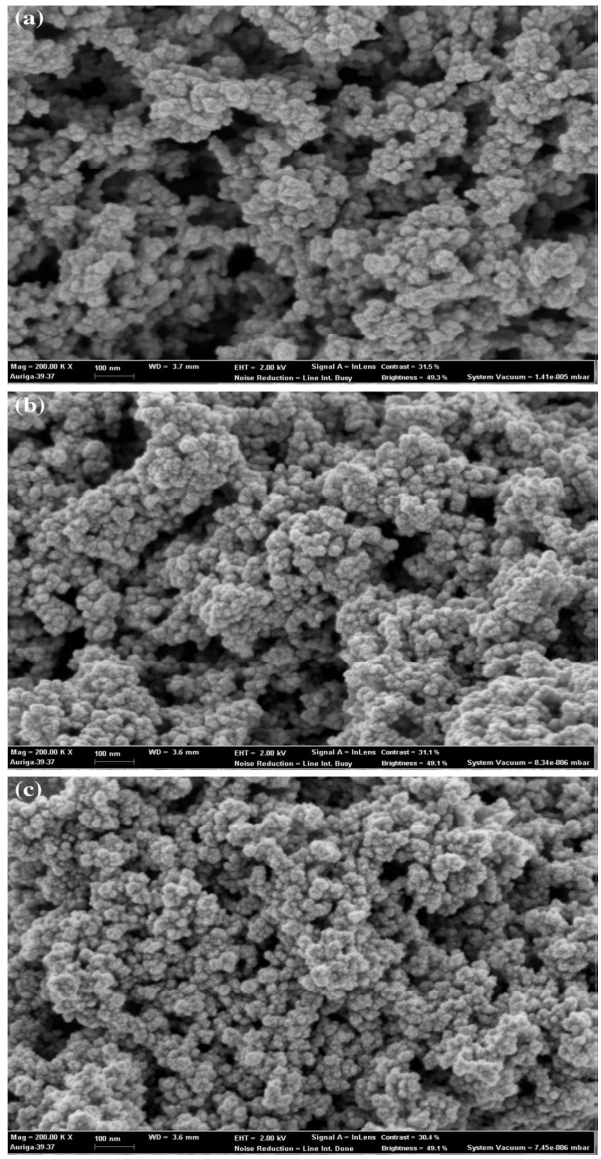

Fig. 4.3 SEM images of sol-gel matrix and immobilized CALB. (a) surface of sol-gel matrix, (b) surface of sol-gel matrix with the activating and cross-linking agents and (c) cross-section of the matrix after covalent immobilization of CALB

4.3.4 Influence of pH on immobilization

Multipoint attachment of enzymes occurs through the interaction of several residues (e.g. amine groups of lysine residues) of the same enzyme molecule and the active groups of the support. Glutaraldehyde has been widely used as cross-linking agent due to its reactivity with the amine groups of the enzymes at around neutral pH. However, the pK_a of lysine residue is about 10.5 so pH is an important parameter in covalent immobilization. In order to intensify the attachment of the lipase, higher pH values were investigated. As shown in Table 4.4, protein is highly immobilized on the activated supports at alkaline pH but there is a reduction on enzyme activity. The decrease in activities of the immobilized enzymes at higher pH values could be due to conformational changes of the enzymes since it is probable that more bonds between enzyme and support were formed.

Table 4.4 Effect of pH value on enzyme attachment and activity using matrix particle size of (–500µm +300µm) and 1.18:2.56 TMOS/ETMS ratio

pH Value	% Attachment	Activity U/g beads	Activity U/g protein
7	80.88	192.02	19.12
8.2	92.95	165.81	14.36
10.5	98.13	104.71	8.59

4.3.5 Stability of the immobilized CALB

Fig. 4.4 shows the residual activity of free and immobilized CALB incubated at 60^{o}C for 24 hours. Soluble enzyme retains about 20% of this activity after 6 hours. However for immobilized CALB, residual activity of about 30% can still be retained after15 hours. This shows that CALB covalently immobilized on sol-gel matrix are more stable than the free enzymes.

The half-life for covalently immobilized enzyme in this study is about 8 hours. The half-life of free enzyme is calculated to be around 3 hours which agrees to the findings of Lee et al. [2010]. Lee et al. [2010] have obtained 4 hours half-life for non-pretreated sol-gel encapsulation of CALB but for oil pretreated, over 80% of the initial activity was retained after 8 hours. The study of Rodriquez et al. [2008], on the other hand using chitosan and agarose to covalently immobilize CALB showed 6.3 hours half-life. From the findings, physical immobilization of enzyme on the surface promotes thermal stabilization.

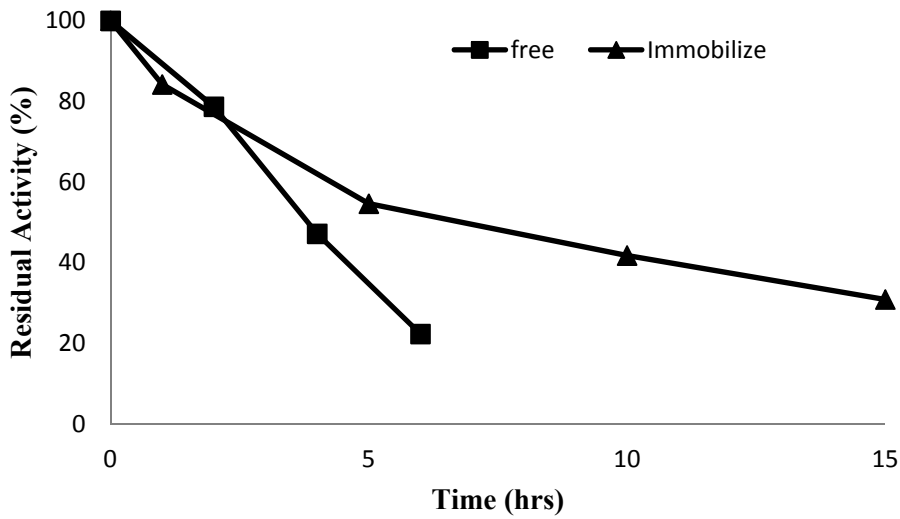

Fig. 4.4 Thermal inactivation profile at 60^{o}C of free (square) and immobilized (triangle) CALB. Experiments were done with enzyme loading of 1242 µg/mL, a particle size of (–500µm +300µm) and 1.18:2.56 TMOS/ETMS ratio

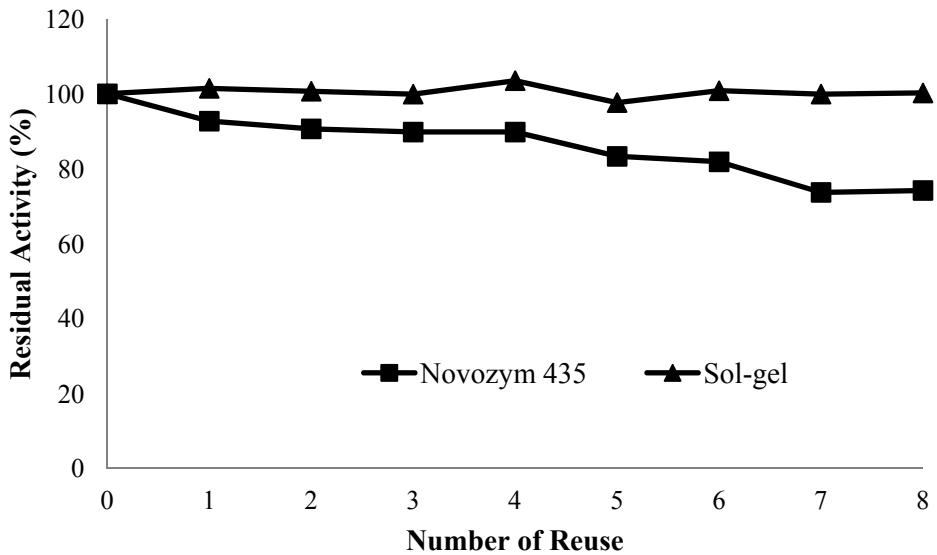

Fig. 4.5 Operational stability of the enzyme immobilization system

This present study is compared to the commercially available Novozym® 435 which immobilized CALB on acrylic resins. The effect of repeated use on the activity of the immobilized CALB is shown in Fig. 4.5. The activity using Novozym® 435 decreased gradually during continues operation process and the immobilized lipase retained about 74% of its initial activity after 8 reuses. However, CALB on the modified sol-gel matrix retained its initial activity even after 8 reuses. Modified sol-gel matrix stabilizes the enzyme and protects the protein from denaturation during the repetitive process.

4.4 Conclusions

A new approach to employ sol-gel materials for enzyme immobilization was studied. Modified sol-gel matrix containing methyltrimethoxysilane and ethyltrimethoxysilane as precursors was used to covalently immobilize *Candida antarctica* lipase B. Improvement on enzyme activity and attachment could be obtained by increasing the enzyme load and decreasing the support's particle size. At alkaline pH, there is a trade-off between degree of attachment and enzyme activity. Immobilizing the enzyme on the modified sol-gel matrix can extend its thermal stability as compared to the soluble enzyme. CALB has high activity on sol-gel matrix with hydrophobic precursor. This approach of

immobilization still provides the enzyme with the appropriate environment while exposing its active site for better accessibility and catalytic efficiency. The sol-gel support material can be further tailored to develop a more suitable immobilized-enzyme system.

Chapter 5

Biodiesel production using the functionally enhanced *Candida antarctica* lipase B immobilized on a modified sol-gel matrix

5.1 Introduction

Microbial lipases constitute an integral part of the manufacturing industries due to their versatility and ease of mass production. Microbial lipases are very attractive for industrial application since their enzymatic properties and their substrate specificity are diverse. With the potential vast application of lipases, newer microbes are being screened, engineering of lipase itself is being investigated, and process conditions and suitable environment are being optimized for enzyme production having desirable characteristics.

Lipases are utilized in detergent industry. The application of lipase in detergent formulation is now common in many countries. In order to improve detergency, heavy powder detergents and automatic dishwashers nowadays usually contain one or more enzymes such as protease, amylase, cellulose and lipase [Ito et al., 1998]. Enzymes are usually selected to reduce the environmental problems associated with plain detergent products and having the ability to withstand relatively harsh washing conditions. Enzymes are advantageous because (a) they can lower the energy requirement by lowering the wash temperature to be used, (b) they are biodegradable thus no harmful residue formation, (c) they allow less harmful chemicals to be added in the detergents, (d) they have no negative impact in the sewage treatment processes,

and (e) they do not present risk to aquatic environment [Hasan et al., 2006]. Novo Nordisk introduced the first commercial lipase, Lipolase, in 1994. It originated from the fungus *Thermomyces lanuginosus* and was expressed in *Aspergillus oryzae*. In the following year, Genencor international also introduced two bacterial lipases, Lumafast from *Pseudomonas mendocina* and Lipomax from *Pseudomonas alcaligenes* [Jaeger and Reetz, 1998]. Researches in developing lipases that will work under alkaline conditions to remove fat stains are being considered these days.

Lipases can also be used to resolve various racemic mixtures. In pharmaceutical industry, chirality is an important factor in the efficacy of many drugs thus the production of single enantiomers of drug intermediates is essentially needed. For instance, profens (2-aryl propinoic acids - an important class of non-steroidal anti-inflammatory drugs (NSAIDs)) are widely used for alleviating the pain and inflammation associated with tissue injury. The pharmacologically active component in profens is the *S*-enantiomers, whereas the *R*-enantiomers often have unwanted physiological side effects and toxicity thus the removal of the *R*-enantiomer of the racemates is desirable. Zhang et al., [2005] utilized the immobilized lipase Novozyme® 435 for the kinetic resolution of racemic flurbiprofen by the method of enantioselective esterification with alcohols.

Lipases are also being used in the production of biofuel, biodiesel. They can catalyze the hydrolysis and the synthesis of esters formed from glycerol and long-chain fatty acids. Some lipases can convert free fatty acids to fatty acids methyl esters even in the presence of high levels of water in which the chemical route of biodiesel production pose some problems. Lipase if immobilized can be easily separated from the glycerol product, thereby eliminating glycerol contamination. Since lipases are biodegradable then it will not present problems on the downstream processing steps such as wastewater treatment.

Other applications of lipases includes the dairy industry in which lipases are used to enhance the flavor of cheeses, accelerate cheese ripening, and the lipolysis of butterfat and cream. Lipases are also utilized in tea processing, pulp and paper industry, cosmetic industry, and as diagnostic tools. The applications of lipases are broadening rapidly and new uses of lipase are still to be explored in these industries. With these several interesting immense applications of lipases, their industrial uses still remains limited due to their high cost of production and low performance in some lipase-mediated reactions.

In this study practical application of lipase on biodiesel production was carried out. CALB enzyme was covalently immobilized on the modified sol-gel

matrix. The feasibility of the functionally enhanced lipase on biodiesel production was investigated.

5.2 Materials and Methods

5.2.1 Materials

Soybean oil was purchased from Ottogi Corporation (Korea). Methyltrimethoxysilane (TMOS), ethyltrimethoxysilane (ETMS) and 3-aminopropyltriethoxysilane (APTS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *Pichia pastoris* strain X-33, the *pPICZαA* plasmid and the QuikChange™ site-directed mutagenesis kit were purchased from Invitrogen (USA). All other chemicals were of analytical grade.

5.2.2 Production of functionally enhanced lipase

Site-directed mutagenesis of CALB was conducted using modified Quikchange™ protocol. Cloning, transformation and expression of CALB variants (e.g. wild type and mutant V139E,A92E) were performed as described in Chapter 3. The CALB variants were concentrated using Amicon Ultra

(Ireland) with a 30-kDa cut-off membrane and purified using the cation exchange chromatography.

5.2.3 Enzyme immobilization

Sol-gel matrix was prepared using 1.18:2.56 (v/v) ratio of TMOS to ETMS to obtain an amphiphilic type of matrix. Particle size that passed through 500µm and retained in 300µm was collected. Surface treatment of the sol-gel matrix was performed as describe in Chapter 4. The treated powders were stored at 4^{o}C until further use.

After enzyme purification, immobilization was carried out at 30^{o}C for 24 hours under gentle stirring. After immobilization, the immobilized enzyme matrix was wash thoroughly with phosphate buffer to remove unbound enzyme. Afterwards, it is dried in a vaccum and stored at 4^{o}C until its next use.

5.2.4 Biodiesel production and analysis

Transesterification reaction was carried out using 1:3 molar ratio of oil to methanol at 45^{o}C and 300 rpm for 24 hours. The alcohol *tert*-butanol was added to increase the miscibility of methanol in the mixture. A volume ratio of

0.5 was used for *tert*-butanol to methanol in the reaction mixture. After reaction, 20 μL of sample was diluted 10X with heptane solution including the internal standard for biodiesel analysis. Biodiesel production was analyzed using Varian 450 gas chromatography with flame ionization detector and HP-5 column. The oven temperature was kept at 50^{o}C for 1 minute, heated to 210^{o}C at a rate of 20^{o}C/min, and then it was maintained at the same temperature for 10 minutes. The temperatures of the injector and detector ports were set at 250^{o}C and 270^{o}C, respectively. Methyl heptadecanoate (Fluka) and methyl linoleate (Sigma) were respectively used as internal and material standard.

5.3 Results and Discussion

As presented in Table 5.1, the characteristic of the CALB enzyme was enhanced when the substrate-binding region was made flexible and the solvent-affecting region was rigidified. The relative hydrolytic activity of the double mutant V139E,A92E using PNPC was improved about two times compared with the wild type. When incubated in methanol for 24 hours, the residual activity of the double mutant was about 2.6 higher than the wild type. In addition, thermostability of the double mutant was also improved as compared with the wild type CALB. This implies that modulating the flexibility of CALB on its different regions can eventually change its characteristics.

Table 5.1 Characteristics of the free CALB enzyme

Enzyme	Relative hydrolytic activity	Residual activity (%) for organic solvent stability in methanol	Thermostability at 60°C	
			Residual activity (%) after 3 hours	Residual activity (%) after 6 hours
Wild Type	1.0	25	42	13
V139E,A92E	2.4	65	54	26

In order to test the immobilization system with an enzyme having enhanced property in a practical application, it was used in the production of biodiesel using soybean oil as a substrate. As shown in Table 5.2, the biodiesel conversion was increased about 4 times after 24 hours when double mutant V139E,A92E was utilized as compared with the wild type CALB enzyme. This increase in biodiesel production could be due to the enhanced characteristics of the double mutant V139E,A92E enzyme. V139E,A92E enzyme has an improvement in hydrolytic activity, organic solvent stability and thermostability as compared with the wild type. The possible synergistic effect of these characteristics in V139E,A92E enzyme is the reason behind its higher biodiesel production.

Currently, the biodiesel conversion using the commercially available Novozyme® 435 is higher than 90% [Gog et al., 2012]. The obtained conversion in this study is only about 37% after 48 hours which is lower compared with Novozyme® 435. Because there are several factors that can affect the degree of transesterification reaction (*e.g.,* reaction temperature, selection of alcohol, the use of solvent, enzyme pretreatment, alcohol to oil molar ratio, water content and the source of lipase), each new preparation of immobilized enzyme system for biodiesel production needs to be optimized for these factors.

Table 5.2 Biodiesel production of the immobilized CALB enzyme

Enzyme	Enzyme Loading (mg/g beads)	Biodiesel Conversion (%)	
		24 hours	48 hours
Wild Type	7.65	8.5	9.8
V139E,A92E	7.08	34.2	37.1

In order to analyze the effect of enzyme activity and stability of the immobilized enzyme system, the percent conversion is plotted with respect to time as shown in Fig. 5.1. After 24 hours, the plots for percent conversion started to plateau for the two enzymes. This could be due to high temperature and/or by the presence of methanol that leads to enzyme denaturation. The difference in conversions between the time intervals of 48 and 24 hours for the wild type and the double mutant are 1.3% and 2.9% respectively. The double mutant has a higher stability compared to the wild type. However, this stability has a minimal effect on the percent conversion of biodiesel compared to the initial rate of conversion. The high conversion obtained by the double mutant V139E,A92E resulted from the inherent high activity of the enzyme which confirms to the discussion presented in Chapter 3. From this, the driving force to have higher conversion at a short period of time is the activity of the enzyme and not the stability.

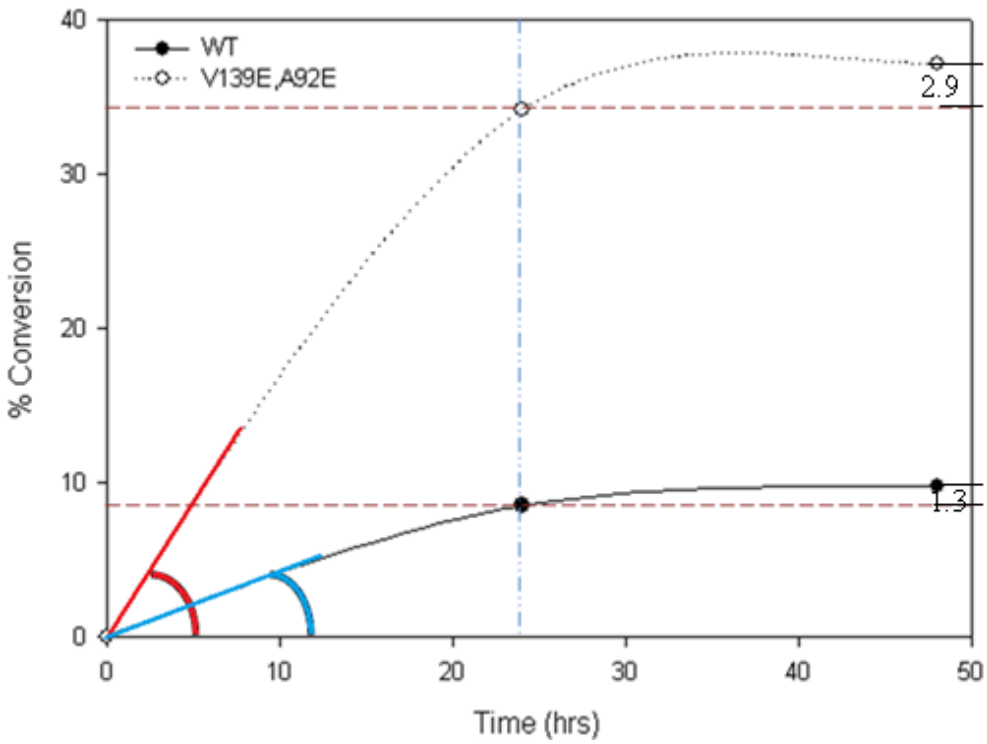

Fig. 5.1 Time course for biodiesel production

5.4 Conclusion

The characteristics of the double mutant V139E,A92E enzyme such as the hydrolytic activity and both thermal and organic solvent stability were enhanced compared with the wild type. The application of the immobilized double mutant (V139E,A92E) can increase biodiesel production. The synthesis of biodiesel using the covalently immobilized double mutant (V139E,A92E) enzyme can be increased by optimizing the operational parameters that affects biodiesel production.

Chapter 6

Overall Discussion and Recommendations

Although *Candida antarctica* lipase B (CALB) does not show an interfacial activation, its activity is increased at a water-lipid interface and when attached to hydrophobic environment. Thus the orientation of CALB is important to determine which site will be mutated for functionality enhancement and to know if immobilization is possible without enormous conformational changes that may affect enzyme activity. For this study, CALB enzyme was divided into two parts (*e.g.,* the substrate-binding region and the hydrophilic solvent-affecting region) using the hydrophobicity profile and its orientation in water-lipid interface (membrane).

The first step of this study is to obtain an enzyme with enhanced catalytic activity. Since flexibility of an enzyme is related to its function such as catalytic activity, the substrate-binding region was made flexible by modulating the flexibility of helix regions near the active site. The flexibility of α5-helix (lid) proves to have a direct relationship with the catalytic function of the enzyme. When the edge of the helix and its near loop residues (residue 139 and 151 respectively) were both mutated to make the lid more flexible, the enzyme activity increases as compared to the wild type and to the single mutant (V139E). This implies that the lid has a functional role on the binding of substrate to the active site of CALB.

The second step of this study is to stabilize the enzyme on organic solvent. The hydrophilic solvent-affecting region was rigidified to obtain an enzyme that resist organic solvent inhibition. Methanol-affecting sites were made rigid by mutations. Generally, double mutations from this study gave higher stability compared to the single mutations and to the wild type. This result confirms that rigidified enzymes possess higher stability.

Normally, the activity increase and the stability enhancement of an enzyme are investigated separately. When the flexibility of the enzyme is modulated to augment its activity it is possible that it will compromise the stability of that particular enzyme. Thus it is important to eliminate this trade-off so that the enzyme can have both stability and higher activity. The next part of this study is to integrate both catalytic efficiency and stability on CALB. Mutants that show higher activity on the substrate-binding regions were paired to the mutants that have higher stability on the solvent-affecting regions. Upon mutant integration, the resultant enzyme mutant (V139E,A92E) possesses both stability and higher activity as compared to the single mutants with enhance activity and stability respectively. This entails that CALB can have both stability and higher activity characteristics by modulating the flexibility of the

hydrophilic solvent-affecting region and the substrate-binding region respectively.

In order to promote the reusability of the enzyme for succeeding applications, the enzyme needs to be immobilized. Among the immobilization techniques, covalent type of immobilization on modified sol-gel supports was applied in this study. The modified sol-gel matrix has an amphiphilic property in which lipase can be activated. Since the enzyme is immobilized on the surface and not encapsulated, mass transfer limitation is reduced. Aside from that, the enzyme during immobilization process is not mixed with the precursors involved in forming the support matrix so that these precursors will not inhibit enzyme activity. Exposed lysine residues of the enzyme were exploited to covalently immobilized CALB. These lysine residues are found on the opposite side of the active site thus keeping the active site well exposed to the substrate and minimizing conformational changes to the enzyme.

The last experimental investigation was the biodiesel production using the functionally enhanced double mutant V139E,A92E enzyme immobilized on the modified sol-gel matrix. The biodiesel conversion was about 3.7 times higher when the functionally enhanced double mutant V139E,A92E enzyme was used compared with the wild type CALB. This higher biodiesel production

was possibly due to the synergistic effect between the higher activity, higher stability in methanol and higher thermostability of the double mutant V139E,A92E enzyme compared with the wild type CALB.

To further improve this study it is recommended to analyze the specificity of the resultant mutants since it is possible that during mutation specificity of the enzyme will be changed. Investigation on double mutants in the substrate binding region of CALB paired or combined with double mutants on the hydrophilic solvent affecting site can be done know the extent of enhancing CALB's functionality by flexibility modulation. Other types of lipases such as *R. oryzae* and *C. rugosa* can also be employed and analyzed using the methods presented in this study since both have lid that covers the active site of the lipase and both enzymes show interfacial activation. Their lid might also play a role on the catalytic activity of *R. oryzae* and *C. rugosa* and modulating its flexibility might increase their catalytic efficiency. Aside from that both lipases has a number of lysine residues that are possible for covalent type of immobilization on the modified sol-gel matrix. The performance of the immobilization system using functionally enhance CALB mutant must be optimized for biodiesel production. Other practical applications can be considered aside from biodiesel production to test the performance of the functionally enhanced immobilized enzyme system.

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국문초록 (Abstract in Korean)

리파제(lipase)는 다양한 공정에서 중요한 역할을 한다. 리파제를 사용하는 공정은 더 적은 에너지를 필요로 하면서도 반응속도가 빠르며 기질선택성이 있기 때문에 저렴한 원료의 사용이 가능하다. 또한 생분해되기 때문에 분리 및 회수 공정이 쉽다는 장점이 있다. 그러나 이러한 장점에도 불구하고 높은 가격과 긴사슬지방산(long chain fatty acid)을 기질로 하는 반응의 낮은 활성, 짧은사슬알코올(short chain alcohol)에 의한 활성 억제 때문에 효소의 수명이 짧아 산업적 응용이 제한된다. 따라서, 해당 생화학 공정이 경제성을 갖추기 위해서는 특성이 향상된 리파제의 개발이 필요하다.

본 연구에서는 효소의 구조적 유연성 조절 및 고정화 방법을 통해 *Candida antarctica* lipase B(CALB)의 기능을 향상시키고자 하였다. CALB 의 역학, 구조 및 작용기를 분석하고 그 유연성을 조절하여 높은

활성과 안정성을 보이는 효소를 얻었다. 또한 효소의 구조적 측면을 고려하여 아미노산 잔기 변이의 효과를 최대화하였다. 작용기, 특히 표면에 노출된 라이신은 해당 효소를 개량된 졸-겔에 고정화하는데 활용되었다.

안정성과 활성을 동시에 증가시키기 위해 CALB 의 다점돌연변이주를 제작하였다. CALB 의 기능을 최적화하기 위해 기질에 결합하는 부위와 친수성 용매에 영향을 받는 부위의 유연성을 조절하는 CALB 변이주를 시험하여 그 결과로 각각 CALB 의 활성과 유기용매 안정성이 증가하는 변이를 찾았으며, 두 부위의 단점돌연변이주들을 조합하여활성과 유기용매 안정성이 동시에 향상된 다점돌연변이주 (V139E, A92E)를 얻었다.

CALB 를 고정화하기 위해 개량된 졸-겔을 활용하는 방법을 연구하였다. 졸-겔 표면의 하이드록시 그룹에 효소를 공유결합하였으며,

소수성 졸-겔 전구체(ethyltrimethoxysilane)를 이용하여 효소의 활성을 증진시켰다. 총 효소량의 80.88%가 고정화되었으며 고정화된 효소의 활성은 192.02U/g of bead 으로 나타났다. 특히 염기성 pH 에서 고정화 비율이 상승하였다. 졸-겔 표면에 효소의 부착 여부는 주사형 전자 현미경을 통해 확인하였다. 졸-겔 표면에 고정화한 CALB 는 60℃에서 반감기가 2.7 배 증가하여 용액상효소보다 높은 열안정성을 보였다. 또한 고정화를 통해 효소를 반복적으로 사용하여도 활성이 유지되었다.

기능이 향상된 효소를 개량된 졸-겔 표면에 부착하여 바이오디젤 생산 능력을 확인하였다. 바이오디젤 생산 능력은 고정화된 야생형 CALB 와 비교해 변이주(V139E, A92E)가 3.7 배 향상되었다. 고정화된 CALB 변이주를 이용한 바이오디젤 생산공정은 최적화를 거쳐 그 생산성을 증가시킬 수 있을 것으로 기대된다. 본 연구에서 사용된 기술은 다른 리파제의 개량에도 적용될 수 있다.

주제어: *Candida antarctica* lipase B, 효소 유연성, 효소 안정성, 돌연변이 유발, 졸-겔, 공유결합-고정화

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